MOLECULAR ANALYSIS OF GENETIC DIVERSITY AND VARIABILITY IN
COLLETOTRICHUM GLOEOSPORIOIDES

BY

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Dedicated
to
Mother

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Abstract of Dissertation Presented to the Graduate School
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MOLECULAR ANALYSIS OF GENETIC DIVERSITY AND VARIABILITY IN
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Results from this study suggest two distinct genetic

subpopulations of *Colletotrichum gloeosporioides* from citrus

based on DNA variation, cultural morphology, and growth.

Type 1 strains are slow growing, morphologically stable,

benomyl tolerant, and contain a single form of ribosomal DNA

(rDNA) as detected by common *Hind*III, *Pst*I, *Sph*I and *Sst*I

fragments hybridizing to cloned *Neurospora crassa* rDNA. Type

2 strains are faster growing, morphologically less stable,

benomyl sensitive, and have rDNA distinct from type 1

strains. The rDNA from type 1 and type 2 strains were cloned

and mapped for 10 restriction enzyme sites and genes coding

for large subunit, small subunit and 5.8S rRNA. A subclone

constructed from the non-transcribed spacer region of type 1

rDNA clone hybridizes only to rDNA from type 1 strains. DNA

polymorphisms detected by heterologous hybridization with

cloned *N. crassa* genes for glutamate dehydrogenase, anthranilate synthetase, histidinol dehydrogenase, and β-tubulin corresponded to type 1 or type 2 strains. All strains liberate free fatty acid from [^{3}H]-labelled cutin and hydrolyze cutin model substrates. Serine esterases from extracellular fluids of cutin-grown *C. gloeosporioides* strains were detected by labelling proteins separated by sodium dodesyl sulfate polyacrylamide gel electrophoresis with ^{3}H-diisofluorophosphate. The two major esterases from type 1 strains have molecular weights of 26 and 20 kilodaltons (kd) whereas the type 2 esterases were 24 and 22 kd. A DNA probe containing the cloned cutinase gene from *C. gloeosporioides* hybridized strongly to DNA from type 2 strains but poorly to type 1 strains. Distinct cutinase genes may be present in the two types of *C. gloeosporioides* strains from citrus. Chromosome-sized DNAs separated by pulsed-field gel electrophoresis corresponded to type 1 or type 2 strains. Type 1 strains had five large chromosomal DNAs 7.6, 7.0, 4.7, 3.7, and 3.3 (or 2.8) million base pairs (Mb) in size and one or two smaller chromosomes (1.6 to 0.63 Mb). Type 2 strains had three large chromosomal DNAs (7.8, 4.7, and 3.7 Mb) and two to four smaller chromosomal DNAs (0.52-0.28 Mb).

CHAPTER 1
INTRODUCTION

Historically, the study of plant diseases dates back to
Theophrastus (371-287 B.C.) who first described disease
conditions of plants, mostly cereal rusts, in *Historia
plantarum* and *De causis plantarum* (Ainsworth 1981).
Afterwards, studies of causal agents of plant diseases and
control measures played a major role in human survival. The
period from the mid-eighteenth to the mid-nineteenth century
was marked by the accumulation of experimental evidence for
the pathogenicity of fungi to plants. Almost all groups of
fungi include some plant pathogenic species but the greatest
number of plant pathogens is to be found among the imperfect
fungi (Ainsworth 1971).

The most important technique for the identification of
plant pathogenic fungi has always been macroscopic and
microscopic morphological examination. Morphology always
took precedence over other considerations in describing
genera and species of plant pathogenic fungi (Ainsworth
1981). The genus *Colletotrichum* Corda was established in
1831 and was characterized by having setose acervuli
containing hyaline, curved fusiform conidia (Baxter et al.
1985). However, there was always confusion in describing

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fungi to this genus due to similar morphological characters of *Vermicularia* Tode and *Gloeosporium* Desm. & Mont. (Dickson 1925; Duke 1928; Arx 1957; Baxter et al. 1985). Duke (1928) suggested that type species of *Vermicularia* and *Colletotrichum* represented the same fungus. Species in the genus *Gloeosporium* probably represent the same fungi as in the genus *Colletotrichum* because the *Gloeosporium* species, which supposedly lack setae, were found to produce them on certain substrates (Baker et al. 1940). Arx (1957) accepted *Colletotrichum* and *Vermicularia* as separate genera while rejecting the more heterogenous genus *Gloeosporium*.

The species concept of *Colletotrichum gloeosporioides* (Penz.) is still uncertain (Van Der Aa et al. 1990). *Colletotrichum gloeosporioides* was first described in 1882 by Penzig as *Vermicularia gloeosporioides*, and in 1887 it was renamed *Colletotrichum gloeosporioides* (Burger 1921). The presence of this fungus in the United States was first observed in 1886 in Florida and was reported by Underwood (1891). Arx (1957) recognized eleven species in the genus *Colletotrichum*, and the name *C. gloeosporioides* with nearly 600 synonyms was maintained to designate the variable anamorph of *Glomerella cingulata* (Stonem.) Spauld. & Schr. He recognized nine forms within the species *C. gloeosporioides*. Arx (1970, 1987) introduced the concept of host forms of *C. gloeosporioides* but did not accept these forms as species or intraspecific taxa with certainty.

Sutton (1980) considered *C. gloeosporioides* a group species showing excessively wide variation.

The conidia of *C. gloeosporioides* are straight, obtuse at the apex, 9-24 x 3-4.5 μm and appressoria are 6-20 x 4-12 μm, clavate or irregular, sometimes becoming complex (Sutton 1980).

Colletotrichum gloeosporioides is a ubiquitous fungus and often causes a variety of diseases commonly known as anthracnose on fruits, leaves and stems of a wide range of host species. The host range of this fungus is so wide that nearly 200 susceptible host species were listed under *C. gloeosporioides* in the Index of Plant Diseases in Florida (Alfieri et al. 1984). Many tropical fruit crops are attacked by this fungus in the field and in post-harvest condition (Nolla 1926; Simmonds 1965; Brown 1975). Citrus is one of the major fruit crops attacked by this fungus, and the diseases of citrus caused by *C. gloeosporioides* have been known since 1886 when it was first isolated from citrus plants in Florida, U.S.A. (Underwood 1891). Rolfs (1904 and 1905) described a group of citrus diseases (wither tip, leaf spot, lemon spot, canker, and anthracnose) caused by *C. gloeosporioides*. A more recently described citrus disease, post bloom fruit drop, PFD (Fagan 1979; Sonoda and Pelosi 1988; McMillan and Timmer 1989) is caused by the same species. The name PFD was suggested by Fagan (1979) to distinguish this disease condition of citrus characterized

by premature fruit drop or blossom blight from normal physiological thinning of fruits. The symptoms first appear as small, brown spots on flower buds or light pink water-soaked spots on open petals. These spots may enlarge and rapidly cover the petals within 24 h. Afterwards, the petals become brown and desiccated. Eventually, young fruitlets become discolored, and they abscise, leaving the calyxes behind as persistent buttons (Figure 1). The disease is economically important in regions where citrus is grown (Denham and Waller 1981; Fagan 1984a). In Florida the disease has been reported from all commercially grown citrus (Sonoda and Pelosi 1988; McMillan and Timmer 1989).

The causal agent of PFD of citrus was identified as *C. gloeosporioides* (Fagan 1979; Sonoda and Pelosi 1988; McMillan and Timmer 1989). *Colletotrichum gloeosporioides* isolated from citrus diseases were reported to be variable in morphology and pathogenicity (Burger 1921). Morphological variability also has been observed in the strains of this fungus causing PFD (Denham and Waller 1981; Sonoda and Pelosi 1988). Three strains *C. gloeosporioides* varying in morphology and pathogenicity were reported to be associated with diseased plants by Fagan (1980). Because of the inconsistency of the morphological characteristics, it is uncertain whether the strains or forms of *C. gloeosporioides* recognized by morphological criteria alone are really different at genetic and molecular level. The morphological

changes could be caused by environmental effects, genetic differences or both. Therefore, study of this group species at the molecular level to understand the genetic and molecular differences among the strains is important.

The present study was undertaken to investigate the morphological variability and potential genetic variation of *C. gloeosporioides* at the molecular level. The objectives of this study are:

1. To examine the basis for morphological variability of *C. gloeosporioides* causing PFD disease of citrus.

2. To investigate genetic variation of *C. gloeosporioides* at the molecular level.

3. To examine variations in chromosome-size DNA and to describe the molecular karyotypes.

Morphological and growth diversity arising from single spore cultures of different *C. gloeosporioides* strains is examined in Chapter 2. Molecular investigations using genetic markers were carried out to study the variation within citrus strains of *C. gloeosporioides*, and they are reported in Chapter 3. Chapter 4 describes the chromosomal variation of *C. gloeosporioides* strains and the molecular karyotypes of the fungus. The results obtained in these studies are reviewed comprehensively in Chapter 5, and a concept of genetically distinct subpopulations of *C. gloeosporioides* is proposed.

Figure 1 Symptoms of post bloom fruit drop disease on sweet orange (*Citrus sinensis* var. *Valencia*) caused by *Colletotrichum gloeosporioides*.

CHAPTER 2
OBSERVED VARIABILITY IN *COLLETOTRICHUM GLOEOSPORIOIDES*
CAUSING POST BLOOM FRUIT DROP IN CITRUS

Introduction

More than six hundred synonyms for the fungal species
Colletotrichum gloeosporioides have been published (Arx
1957). This reflects the considerable amount of diversity
and variability observed for the fungus by different
investigators throughout the world. Sutton (1980) could not
give a standard morphological description of *C.
gloeosporioides*. He considered the different forms of this
fungus to be within a group species.

The association of *C. gloeosporioides* with citrus dates
back to 1886-1891 (Underwood 1891). The fungus has been
found to cause various diseases on this crop for the past
century (see Chapter 1). The most thorough studies on the
morphological variation of *C. gloeosporioides* were done by
Burger (1921). The fungus he studied was the causal agent of
bloom drops and leaf spots in citrus. Cultural
characteristics such as mycelial color, growth and
sporulation enabled him to classify *C. gloeosporioides*
strains into five groups. However, some strains did not fit
into any of the morphological classes due to inconsistency

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of mycelial and sporulation characteristics in continuous culture.

Mycelial sectors distinguished by growth and color differences within single spore cultures of strains are another type of variability observed in *C. gloeosporioides*. Burger (1921) observed black and white mycelial sectors in single spore cultures of the fungus. When single spored, these black and white sectors were able to maintain their identity in continuous culture.

Burger (1921), after studying cultural characteristics, spore dimensions, and sectoring, concluded that *C. gloeosporioides* is constantly giving off new types under natural conditions as well as in artificial cultures. He further suggested that these variabilities of *C. gloeosporioides* may have arisen from environmental effects as well as from high frequency mutations.

Morphological and pathogenic variability in *C. gloeosporioides* causing PFD of citrus has been reported by Fagan (1979,1980) and Denham and Waller (1981). Three different forms of *C. gloeosporioides* were recognized by Fagan (1980). Two forms, *cgm* with gray to dark gray mycelium and *cgc* with light gray mycelium, were isolated from senescent leaves and were nonpathogenic to citrus flowers. The pathogenic form, *cgp*, had off-white to pink mycelium and was isolated from floral parts of citrus. Fagan (1980) concluded that at least two strains of *C. gloeosporioides*

causing PFD occurred in Belize. These strains corresponded to morphological groups of *C. gloeosporioides* described by Burger (1921).

The objective of this study is to examine the morphological and phenotypic diversity of *C. gloeosporioides* causing PFD of Tahiti lime (*Citrus aurantifolia* Swingle) and Sweet orange (*Citrus sinensis* Osbek).

Materials and Methods

Strains of *Colletotrichum gloeosporioides*

Strain number, host, place and year of isolation are tabulated in Appendix A. Isolation of *C. gloeosporioides* from host plants was carried out as follows. Host plant tissues were surface sterilized in 1% sodium hypochlorite (Clorox Co., Oakland, CA) for 30-60 s, rinsed 3 times with sterilized water and plated on potato dextrose agar (PDA, Difco laboratories, Detroit, MI) plates. Edges from growing mycelia were isolated and maintained in the laboratory as strains. Strains were grown in 20% (w/v) V-8 juice (Campbell Soup Co., Camden, NJ) for 7 d at 250 rpm on a Lab-Line orbit shaker (Lab-Line Instruments Inc., Melrose Park, IL). Spores were collected by centrifugation at 7000 x g for 5 min and washed 2 times with sterilized water before storing in 50% glycerol (in water) at -80°C. To obtain single spore cultures, spores were spread on PDA plates; 14-16 h later, germinating spores were isolated under a dissecting

microscope (25x10 magnification) and plated on PDA plates. Morphology of colony growth, mycelial color and sectoring were examined in PDA culture and still liquid culture, potato dextrose broth (PDB Difco laboratories, Detroit, MI).

To examine the nuclear number, spores were stained with 1% aniline blue (Sigma Chemical Co., St. Louis, MO) in 50% glycerin in water (Tu and Kimbrough 1973). To stain nuclei, a drop of spores in water was placed on a microscopic slide, and a drop of stain was added. The slide was then heated over a flame for 5-10 seconds. Approximately 1000 spores were examined for each strain.

Pathogenicity

All the strains were tested for their ability to infect flowers of Tahiti lime under natural conditions in the field as well as in the laboratory. Strains were grown in 20% V-8 juice for 7 days and inoculum containing 10^7 spores ml^{-1} water were prepared. Tahiti Lime flowers were sprayed using a hand sprayer to wetness with inoculum or water, and symptom development was observed for 3 days. Each treatment contained 10-15 flowers. The control was sprayed with water.

Benomyl Tolerance

The growth of *C. gloeosporioides* strains was examined in PDA medium containing 0, 2 and 10 μg benomyl (methyl-(butyl carbamoyl)-2-benzimidazolecarbamate, Sigma Chemical

Co., St. Louis, MO) ml^{-1}. Radial growth of the mycelial

colony was measured every 24 h for a 10 day period. Growth

rates in mm h^{-1} were estimated by the slopes obtained with

linear regression analysis of the growth curve. A comparison

of slopes was made using analysis of variance (Appendix B).

Each treatment was replicated 5 times, and the experiment

was repeated once with 2 replicates.

Results

Colletotrichum gloeosporioides Strains from Citrus are Morphologically Variable

The *C. gloeosporioides* strains examined can be grouped

into two major categories based on morphology and growth

characteristics. Type 1 strains (H-1, H-3, H-9, H-21, H-22,

H-25B, H-36, IMB-3, LP-1, Maran, OCO, and TUR-1) produce

morphologically stable and relatively slow-growing mycelial

colonies in PDB. The colonies are orange-colored and have

appressed mycelia with abundant sporodochia (Figure 2.1).

Type 2 strains (H-4, H-11, H-12, H-23, H-24, H-46, H-47, H-

48, 180269 and 226802) grow faster and produce mostly gray,

fluffy mycelial colonies (Figure 2.1). The type 1 strains

grow at a significantly slower rate from 0.008 to 0.10 mm

h^{-1}; type 2 strains grow significantly faster from 0.12 to

0.15 mm/ h^{-1} as calculated by slopes of linear regression

data (Table 2.1). The strain types also differ in culture

Table 2.1   Effect of benomyl concentration on the estimated
radial growth rates in mm h^{-1} of *Colletotrichum
gloeosporioides* type 1 and type 2 strains.

Strain	Benomyl concentration μg/ml		
	0	2	10
Type 1			
H-1	0.10	0.033	0.029
H-3	0.10	0.033	0.033
H-9	0.10	0.045	0.041
H-25B	0.041	0.041	0.041
H-36	0.095	0.037	0.033
IMB-3	0.008	0.008	0.004
LP-1	0.10	0.050	0.041
Maran	0.041	0.029	0.033
OCO	0.095	0.037	0.033
Type 2			
H-4	0.141	0.00	0.00
H-11	0.125	0.00	0.00
H-12	0.121	0.00	0.00
H-46	0.133	0.00	0.00
H-47	0.133	0.00	0.00
H-48	0.145	0.00	0.00
180269	0.133	0.00	0.00
226802	0.150	0.00	0.00

stability as determined by their ability to produce sectors of different color, morphology and growth habit. To quantitate these levels of instability, 100 conidia were isolated from three type 1 strains and two type 2 strains and tested for morphological stability. One hundred single spore cultures from strains H-1, H-3, and H-25B (type 1) grown in PDB were found to produce identical colonies. One hundred single spore cultures from strain H-12 and H-48 (type 2) produced 100% sectoring colonies. These colonies varied in colony color from dark gray to gray, white, and orange with different growth rates (Figures 2.2, 2.3, and 2.4). Sporodochia production was scattered or inhibited but could be stimulated by mycelial injury (Figure 2.5). One hundred injury-induced spores from an H-48 gray mycelial sector produced 50 sectoring colonies, 24 dark gray with no sporodochia and 26 orange colonies with scattered sporodochia production.

Colletotrichum gloeosporioides Strains have Different Nuclear Numbers in their Spores

The nuclear number observed by aniline blue staining varied from 1 to 3 per single spore (Table 2.2). All spores examined from all isolates were single celled. All the isolates examined contained spores with more than one nucleus as identified by dark stained objects distinguished from the lightly stained cytoplasm under the high

Table 2.2 Percentage of spores carrying different numbers of
nuclei in *Colletotrichum gloeosporioides* strains.

Strain	Percentage of spores* Number of nuclei		
	1	2	3
H-1	92.6	6.8	0.6
H-3	97.8	2.2	<0.1
H-4	94.3	5.4	0.3
H-9	96.4	3.4	0.2
H-12	98.8	1.2	<0.1
H-25B	96.9	2.9	0.2
H-46	93.4	6.0	0.6
H-48	92.8	6.6	0.6
180269	92.9	6.6	0.5
226802	94.7	5.2	0.1
LP-1	99.6	0.4	<0.1
Maran	98.6	1.4	<0.1

*=Calculated from 1000 spores

magnification (46x10) of a light microscope. Spores
containing a single nucleus varied from 92.6 to 99.6% in the
15 isolates studied. The maximum number of nuclei observed
within a single spore was 3, and the percentage of spores
containing three nuclei varied from <0.1-0.6%. The
percentage of spores containing two nuclei varied from 0.4-
6.8%.

Both Type 1 and Type 2 Strains are Pathogenic to Tahiti Lime Flowers

Brown lesions developed in flowers individually
inoculated with all strains of the pathogen 24 h after
spraying. The petals were blighted completely at 36 h and
had dropped at 48 h. Flowers sprayed with water alone were
not blighted after 72 h. The fungal strains reisolated from
infected tissues were found to be morphologically like the
original strains. The relative virulence of strains was not
measured in this study.

Type 1 and Type 2 Strains Differ in their Tolerance to Benomyl

All type 2 strains were completely inhibited by 2 or 10
μg ml^{-1} benomyl in PDA, but type 1 strains were more
tolerant. Average growth rates for individual type 1 and
type 2 strains are listed in Table 2.1. Analysis of variance
showed that benomyl concentration had a significant effect
on type 1 strains. There was a significant interaction

between strains and concentration indicating that growth rate of each strain may respond differently to different concentrations of benomyl (Appendix B).

Discussion

Grouping of *Colletotrichum gloeosporioides* strains based on morphological and physiological observations was first attempted by Burger (1921). However, morphologically based groups of *C. gloeosporioides* strains have been inconsistently described in this and subsequent studies (Burger 1921; Arx 1957; Sutton 1980). Fagan (1980), Denham and Waller (1981), and Sonoda and Pelosi (1988) reported morphological variations associated with this fungus isolated from Sweet orange cultivars. The type 1 strains in this study show similarities in morphology, growth and sporodochia production to strains designated *cgp* by Fagan's (1980) description and correspond to the orange colored, slow-growing colonies described by Sonoda and Pelosi (1988). The more variable type 2 strains show similarities to the *cgm* and *cgc* strains of Fagan and correspond to faster growing colonies described by Sonoda and Pelosi.

Both type 1 and type 2 strains were isolated from sweet orange (*C. sinensis*) as well as Tahiti lime (Appendix A). Both types were pathogenic to Tahiti lime flowers as

Figure 2.1. Morphology of type 1 (left) and type 2 (right)
strains of *Colletotrichum gloeosporioides* grown
in potato dextrose broth.

Figure 2.2 Fluffy and appressed mycelial sectors
produced by a single spore culture of
Colletotrichum gloeosporioides type 2 strain in
potato dextrose agar.

Figure 2.3. Dark gray, light gray and orange mycelial
sectors produced by a single spore culture of a
Colletotrichum gloeosporioides type 2 strain in
potato dextrose broth.
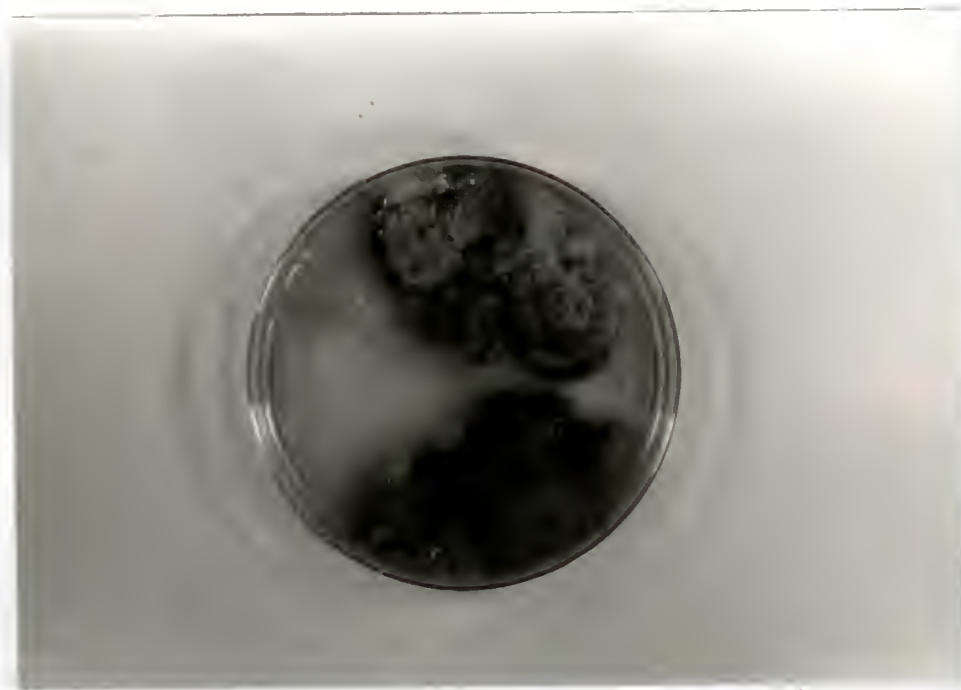
Figure 2.4. Multi-colored mycelial sectors produced by a
single spore culture of a *Colletotrichum
gloeosporioides* type 2 strain in potato dextrose
broth.

Figure 2.5. Mycelial injury can induce type 2 (non-sporodochia-forming) type 2 strains to form sporodochia.

indicated by inoculation tests, confirming previous results
(Sonoda and Pelosi 1988). Variation in the ability of type 1
and type 2 strains to cause disease on Tahiti lime flowers
was not observed. However, only a single high inoculum
concentration was used for pathogenicity testing.
Differences in virulence perhaps could be found if a
dilution series of inoculum were used in assessing the
disease-causing potential of the strains. Sonoda and Pelosi
(1988) and Agostini et al (1992) suggested that slowly
growing, orange-colored strains (type 1 strains) were the
actual causal agent of PFD because only they could be
consistently isolated from diseased petals in the field
while the gray (type 2) strains were isolated primarily from
leaves, stems and fruit. This observation has been confirmed
by other workers (Agostini et al. 1992; Gantotti and Davis,
personal communication). Clearly, further pathogenicity
testing and field sampling are needed to confirm whether one
or both types of the pathogen are important in PFD
epidemics.

The genetic and molecular basis of the morphological
diversity caused by sectoring of *C. gloeosporioides* is yet
to be elucidated. One of the probable genetic explanations
for the observed morphological diversity and sectoring of
type 2 strains may be heterokaryosis followed by
parasexuality or nuclear sorting out. Heterokaryosis and
parasexuality have been found to contribute to the variation

of *C. gloeosporioides* as reviewed by Baxter et al. (1985).
The strains studied carried only <7% spores with 2-3 nuclei.
Therefore, heterokaryosis or nuclear sorting out may not be
a cause of 100% sectoring observed in type 2 strains.
Multinuclear spores have been reported in *C. gloeosporioides*
as well as many other fungi (Panaccione et al. 1989; Shirane
et al. 1989; TeBeest et al. 1989). The multinuclear
condition may arise from division of the nucleus without
division of the spore (Churchill 1982). Hence, it may
represent homokaryotic condition.

An interesting phenomenon observed between type 1 and
type 2 strains is the differential sensitivity to benomyl
(methyl-2-benzimidazole carbamate: active ingredient in the
commonly used fungicide benlate). Type 2 strains were
completely inhibited by the levels of benomyl tested while
type 1 strains were tolerant although their growth rates
were significantly reduced (Table 2.2). The benomyl
tolerance of type 1 strains may have practical consequences
to the control of this disease. Current control measures
include spraying benomyl to control PFD (Fagan 1984b). If
type 1 strains are the primary causal agent of PFD as
previously suggested (Sonoda and Pelosi 1988; Agostini et
al. 1992; Gantotti and Davis, personal communication),
spraying in the field may only partially inhibit the
virulent pathogen while completely eliminating the less

virulent form. While slowing the epidemic in the short run, this practice may have the long-term effect of selecting for the most virulent form of the fungus.

CHAPTER 3
DNA POLYMORPHISMS FOUND AT MANY GENETIC LOCI EXAMINED
IN *COLLETOTRICHUM GLOEOSPORIOIDES*

Introduction

Ribosomal DNA in Fungi

The nuclear ribosomal RNA (rRNA) genes of eukaryotes
are clustered in tandemly repeating units known as ribosomal
DNA (rDNA) unit repeats. In fungi as in many other
eukaryotes, each rDNA unit repeat consists of coding regions
for small subunit, SSU (17-18S), 5.8S, and large subunit,
LSU (25-26S) rRNA and intervening internal and external
transcribed and non-transcribed spacer (NTS) regions
(Fedoroff 1979; Chambers et al. 1986). Each rDNA unit repeat
codes for a 35S rRNA precursor which gives rise to SSU and
LSU rRNAs. In *Neurospora crassa* and *Saccharomyces cerevisiae*
a 35-37S rRNA precursor cleaves into a 17-18S rRNA of the
small ribosomal subunit (37S), and the 5.8S and 25S rRNAS of
large ribosomal subunit (60S) required for building 80S
ribosomes (Russell et al. 1976; Bell et al. 1977; Planta et
al. 1980).

The number of times rRNA genes are repeated varies
depending on the species of the organism. It was estimated

that there are about 185-225 copies of rDNA unit repeats in *Neurospora crassa* (Krumlauf and Marzluf 1980; Rodland and Russell 1982), 140 copies in yeast, *Saccharomyces cerevisiae* (Schweizer et al. 1969; Rubin and Sulston 1973), 59 copies in *Rhizoctonia solani* (*Thanatephorus praticola* AG-4) (Vilgalys and Gonzalez 1990) and 60-90 copies in *Coprinus cinereus* (Cassidy et al. 1984) per haploid genome.

Another rRNA gene recognized in fungi is 5S rRNA gene. The 5S rRNA gene may be present within the rDNA unit repeat or dispersed elsewhere in the genome. The 5S rDNA sequences are located within the same rDNA unit repeat in *S. cerevisiae* (Bell et al. 1977), *Mucor racemosus* (Cihlar and Sypherd 1980), *S. rosei and S. carlsbergensis* (Verbeet et al. 1983), *C. cinereus* (Cassidy et al. 1984), *Schizophyllum commune* (Buckner et al. 1988), *R. solani* (Vilgalys and Gonzalez 1990), the slime mold, *Dictyostelium discoideum* (Maizels 1976), and water mold, *Achlya ambisexualis*, (Rozek and Timberlake 1979). It is located elsewhere in the genome in *N. crassa* (Free et al. 1979; Selker et al. 1981), *Schizosaccharomyces pombe* (Tabata 1981), *Aspergillus nidulans* (Borsuk et al. 1982), yeasts, *Yarrowia lipolytica*, (Van Heerikhuizen et al. 1985), and *Cochliobolus heterostrophus* (Garber et al. 1988).

In all known cases the 5S rRNA is transcribed independently as a primary transcript separate from the 35-37S rRNA precursor transcript (Udem and Warner 1972;

Miyazaki 1974). When the 5S rRNA gene is within the same
unit repeat the 5S rRNA gene could be located in the same
strand, transcribed in the same direction as the other rRNA
genes or in the opposite strand, and transcribed in an
antiparallel manner (Aarstad and Oyen 1975). In *C. cinereus*
the 5S rRNA gene is transcribed in the same direction as the
rest of the rRNA genes (Cassidy et al. 1984).

Ribosomal DNA is Polymorphic in Many Fungi

Ribosomal DNA is a unique genetic marker which can be
used in the study of relatedness among organisms. Generally
the number of rDNA unit repeats is maintained from
generation to generation of an organism. The meiotic
recombination is suppressed within the rDNA array. In *N.
crassa* (Russell et al. 1988) and in *C. cinerus* (Cassidy et
al. 1984) the rDNA was shown to be inherited in a simple
stable Mendelian fashion exhibiting an approximately 1:1
ratio of the two parental rDNA types. No meiotic
recombinants were detected among the progeny indicating that
non-sister chromatid crossing over was highly suppressed in
the rDNA region of these organisms. However, Butler and
Metzenberg (1989 and 1990) demonstrated that *N. crassa* rDNA
can undergo unequal sister chromatid exchange and that the
number of rDNA unit repeats does not segregate in a simple
Mendelian fashion. Their observations suggested that

although the same rDNA RFLP can be inherited, the number of unit repeats can be different from either of the parents.

Within a given species, the members of the rRNA gene family are reasonably homogeneous in sequence, as are their associated spacer sequences, despite frequent length and restriction site differences among the latter. Yet there are interspecific differences in sequence and these appear to be much more pronounced for spacers than for genes. Smith (1973) suggested that the differences between genes and spacers might be in the rate at which they accumulate mutations. Chromosomes containing mutations deleterious to gene function would be eliminated by natural selection while neutral spacer mutations would be retained in the population. Hence, spacers change more rapidly than genes simply by retaining a larger fraction of mutation (Smith 1973).

Length heterogeneity and restriction site polymorphisms in rDNA has been commonly observed in many fungi. These polymorphisms were common among strains of *S. cerevisiae* (Petes and Botstein 1977), *N. crassa* (Russell et al. 1984), *S. commune* (Specht et al. 1984), *C. cinerus* (Wu et al. 1983), and *Y. lipolytica* (Clare et al. 1986). Both restriction site and length polymorphisms have been also observed among biological species of *Armillaria* (Anderson et al. 1989). Polymorphisms of rDNA in many fungi are located within the NTS region of the rDNA unit repeat (Cassidy et

al. 1984; Van Heerikhuizen et al. 1985; Rogers et al. 1989).
Chambers et al. (1986) compared the 8.4 kb rDNA unit repeat
of *N. intermedia* and *N. sitophila* with the 8.7 kb long rDNA
unit repeat of *N. crassa* and found that the 300 bp
difference was within the NTS region. Verbeet et al. (1983),
comparing *S. rosei* and *S. carlsbergensis* by heteroduplex
analysis, concluded that the NTS regions are largely non-
homologous in sequence whereas the transcribed regions are
essentially homologous. Russell et al. (1984) studied the
organization of the rDNA unit repeat in the strains of *N.
crassa*, *N. tetrasperma*, *N. sitophila*, *N. intermedia*, and *N.
discreta* and found that the size of the unit repeat has been
highly conserved among the strains of *Neurospora*. However, a
restriction enzyme site polymorphism in the NTS region was
found between the strains. This restriction site
polymorphism was strain-specific and not species-specific.
Restriction enzyme mapping of rDNA in yeast, *Kluyveromyces*
species has shown a length variation, and the variability
was found to reside in the NTS region (Lachance 1989).
Martin (1990) reported the presence of restriction site and
length polymorphisms within single oospore isolates of the
Oomycete genus *Pythium*, and the differences were found
within the NTS region and 3' end of the 26S coding region.
The NTS region has also been useful to study the
phylogenetic relatedness among fungal species and other
organisms (Verma and Dutta 1987).

The transcribed intergenic spacer (ITS) has also been shown to be variable in fungi. In *S. commune* location of strain-dependent length polymorphisms resided in the ITS region between 18S and 5.8S cistrons (Buckner et al. 1988). Chambers et al. (1986) compared the sequences of ITS regions for *N. crassa* and *S. carlsbergensis*, and found that there is a general lack of homology between the internal transcribed spacer regions between 5.8S and 26S rRNA genes of these two species. Buchko and Klassen (1990), using PCR technique to amplify the ITS region, demonstrated length heterogeneity in strains of *Pythium ultimum*.

The locations of rDNA polymorphisms were not confined to the ITS regions. Polymorphisms within the coding regions of rRNA genes due to addition or deletion of restriction enzyme sites were found in fungi (Chambers et al. 1986).

Another cause of rDNA polymorphism in eukaryotes is the presence of introns in the coding regions. In fruit fly, *Drosophila melanogastor*, the presence of an intron in the coding region of the 28S rRNA gene has given rise to polymorphism (Glover and Hogness 1977) of rDNA in this organism. In fungi there are no conclusive reports for the presence of introns in rRNA genes. However, Buckner et al. (1988) examining the strain-dependent rDNA length polymorphism in *S. commune* suggested the possibility of having an intron in the coding region of the 18S rRNA gene.

Deletions of large fragments of rDNA may also occur in organisms as reported by Malezka and Clark-Walker (1989). A deletion of a 300 kb chromosomal fragment containing 35-40 rRNA cistrons has given rise to a new petite positive strain of *Kluyveromyces lactis*.

One of the objectives of this study is to investigate the variation of rDNA among the strains of *C. gloeosporioides* causing PFD.

Fungal Cutinase Genes and Cutinase Isozymes

Plant pathogenic fungi penetrate their hosts through the cuticle of epidermal cells or through cutinized cells below natural apertures. Penetration may take place by mechanical pressure (Brown and Harvey 1927; Brown 1936; Pristou and Gallegly 1954; Chakravarty 1957; Wood 1960; Meredith 1964; Bonnen and Hammerschmidt 1989b), or by enzymatic degradation of the cuticle (De Bary 1887; Miyoshi 1895; Linskens et al. 1965; Akai et al. 1968; Kunoh and Akai 1969; Shayakh et al. 1977; Kolattukudy 1985) or by both (Ellingboe 1968; Shishiyama et al. 1970; Nicholson et al. 1972).

The plant cuticular barrier is composed of a biopolymer, cutin and associated waxes providing a protective covering against pathogen invasions and hazardous effects of environment (Martin and Juniper 1970). The structure of cuticle varies from one plant to another, and

it is influenced by genetic background as well as
environmental factors (Martin and Batt 1958; Martin 1964).
Almost all parts of the plant, surfaces of epidermal cells
of aerial plant parts, substomatal areas, mesophyll and
palisade cells (Martin and Juniper 1970; Sitholey 1971),
flower parts, seed coat (Kolattukudy et al. 1974) fruit
(Espelie et al. 1980), roots and tubers (Kolattukudy and
Agrawal 1974; Kolattukudy et al. 1975) contain a cuticular
layer.

The biopolymer, cutin is composed of C_{16} and C_{18} hydroxy
and hydroxy epoxy fatty acids (Van den Ende and Linskens
1974; Espelie et al. 1980; Kolattukudy 1980, 1981). The
composition of the cutin polymer and the proportions of C_{16}
and C_{18} fatty acid monomers may vary depending on plant
species or varieties, organs of the same plant, or on growth
conditions (Espelie et al. 1979; Kolattukudy 1980).

The enzyme, cutinase can facilitate the hydrolysis of
cutin into its components (Baker and Bateman 1978; Dickman
et al. 1982). These hydrolysis products of cutin are also
potent inducers of the cutinase gene of the penetrating
fungus (Woloshuk and Kolattukudy 1986). A small amount of
cutinase is constitutively expressed in the fungal spore
which senses the contact with the plant cuticle via the
unique cuticle monomers generated by this small amount of
cutinase. Consequently, these monomers trigger the
expression of the cutinase gene/ genes needed for the

production of cutinases which eventually degrade the cuticle (Köller et al. 1982; Kolattukudy 1985; Woloshuk and Kolattukudy 1986; Podila et al. 1988; Kolattukudy et al. 1989).

Many plant pathogenic fungi examined have shown production of different levels of cutinase isozymes (Purdy and Kolattukudy 1975a; Lin and Kolattukudy 1980; Kolattukudy et al. 1981). Direct observational, enzymological and histochemical evidences have suggested that cutinase is essential for the penetration of the plant by the fungal pathogens. Specific antibodies prepared against cutinase from *Nectria haematococca* (*Fusarium solani* f. sp. *pisi*, Shaykh et al. 1977) and/ or diisopropylfluorophosphate (DFP), a potent inhibitor of serine esterases, can prevent infection of the host by this fungus indicating that cutinase plays an essential role in the infection process (Kolattukudy 1979). Dickman and Patil (1986) obtained cutinase-deficient mutants of *C. gloeosporioides*, the causal agent of papaya anthracnose, and found that they were nonpathogenic to the intact papaya fruit. However, these cutinase-deficient mutants produced normal lesions when papaya surfaces were artificially wounded or treated with purified cutinase enzyme. Dickman et al. (1989) were able to introduce a *Fusarium* cutinase gene into a wound pathogen, *Mycosphaerella* species through genetic transformation. These transformants acquired the capacity to infect intact papaya

fruits, and the infection by them was prevented by the treatment of antibodies against *Fusarium* cutinase.

Cutinolytic enzymes have been purified and characterized from various plant pathogens (Kolattukudy 1980, 1985; Köller 1991) including *Colletotrichum gloeosporioides*. The single enzyme produced by a strain of *C. gloeosporioides* isolated from papaya fruit had a molecular weight of 24 kd (Dickman et al. 1982) which is very similar in size to other fungal cutinases (Kolattukudy 1980, 1985; Köller 1991).

There is considerable heterogeneity of molecular, immunological and enzymological properties and primary sequences of the cutinase enzymes (Kolattukudy 1985; Ettinger et al. 1987; Trail and Köller 1990). Sequence comparison of the cutinase genes cloned from *C. gloeosporioides* and *N. haematococca* revealed considerable dissimilarity. Even though both cutinase genes shared homologous regions critical for activity and structural integrity, only 43% of the amino acids were directly conserved (Ettinger et al. 1987). Profound differences in cutinase appear to exist even among *Colletotrichum* species (Kolattukudy 1987). A cDNA clone of the cutinase gene from *C. capsici* hybridized to genomic DNA from *C. graminicola* and *C. gloeosporioides*, but not with *C. orbiculare* (syn. *C. lagenarium*) or *C. coccodes* DNA. Though not extensively investigated, the phenomenon of cutinase diversity is also

reflected in enzyme kinetics and activity. For example, cutinolytic activity of esterases purified from *N. haematococca* (Purdy and Kolattukudy 1975b) and *F. roseum culmorum* (Soliday and Kolattukudy 1976) was highest at alkaline conditions (pH 10), whereas an optimum of pH 6.5 was determined for the enzyme derived from *Venturia inaequalis* (Köller and Parker 1989). Baker and Bateman (1978) assayed sixteen plant pathogenic fungi, *Botrytis cinera, B. squamosa, Cladosporium cucumerinum, C. graminicola, N. haematococca, F. roseum, Gloeocercospora sorghi, Helminthosporium carbonum, H. maydis* (race T), *Pythium aphanidermatum, P. arrhenomanes, P. ultimum, R. solani, Stemphylum loti,* and *Sclerotium rolfsii* and found that they can produce various levels of cutinase isozymes with acidic or alkaline pH optimas. Evidence has been presented that these differences in enzymatic properties may allow for the tissue specificity of pathogens. Trail and Köller (1990) reported an acidic pH optimum for the leaf pathogen, *Cochliobolus heterostrophus*, pH 6.5 and an alkaline pH optimum for the stem pathogen, *R. solani*, pH 8.5. The leaf and stem pathogen, *Alternaria brassicola*, produced two cutinases, one with acidic and the other with alkaline pH optima, pH 7.0 and 9.0 respectively. Differences also have been reported for the cutinases produced by *N. haematococca* and *C. gloeosporioides*. Only the enzyme from the latter accepted palmitate as a substrate and the

specific esterase activity with both *p*-nitrophenol butyrate and polymeric cutin was reported to be substantially lower (Dickman et al. 1982).

The ability of a pathogen to produce cutinase can be used to measure the infecting capacity of the fungal pathogen (Dickman et al. 1982; Köller *et al.* 1982). Thus the regulation of expression of the cutinase gene could be highly relevant to pathogenesis. Therefore, the cutinase gene may be a good genetic marker to examine polymorphisms among populations of fungi with differing specificities and capabilities of causing plant disease.

One of the goals in this study was to investigate if differences in morphologically defined type 1 and type 2 strains (see Chapter 2) are also reflected in the cutinase isozymes and genetic organization of cutinase gene or genes.

Restriction Fragment Length Polymorphisms (RFLP) in Fungi

When fungal nuclear DNA is digested with a restriction enzyme an enormous number of fragments generally result. In order to study the restriction fragment pattern of DNA from a specific chromosomal locus these fragments are size fractionated by gel-electrophoresis, and individual fragments are identified by Southern hybridization to labelled probes (Southern 1975; Bernatzky 1988). Each restriction fragment that hybridizes to a given probe constitute a discrete chromosomal locus. Alleles can be

differentiated by the variation in restriction sites.
Restriction fragment length polymorphisms result from
specific differences in DNA sequence such as single base
pair substitution, additions, deletions, or chromosomal
changes (inversions and translocations) that alter the
fragment size obtained by restriction enzyme digestion.
First demonstrated by Grodzicker et al. (1974) for mapping
temperature-sensitive mutations in adenovirus, RFLP analysis
has contributed significantly in genetic analysis of many
organisms.

Genetic studies of plant pathogenic fungi have been
difficult due to lack of easily assayed genetic markers.
Restriction fragment length polymorphism has become a
popular tool for studying genetics of fungi because RFLP
markers are precise, codominant, selectively neutral, easy
to assay, and provide an unlimited number of genetic markers
(Michelmore and Hulbert 1987). Restriction fragment length
polymorphism could provide sufficient markers for the
development of detailed linkage maps for the plant
pathogenic fungi. It is also useful in studying genetic
variation, genomic organization and population genetics of
fungi. Combined with pulsed field gel electrophoresis RFLP
analysis provides a powerful tool to monitor genetic changes
throughout the genome (Michelmore and Hulbert 1987).
Analysis of RFLP markers that flank genetic loci such as
virulence genes can provide information on the genetic basis

of any changes in phenotype. Closely linked RFLPs can be used as tags for important traits. With RFLP markers it is possible to create a molecular fingerprint of specific individuals in a population. Hence, RFLPs provide a tool for studying asexually reproducing populations of fungi. Engels (1981) and Hudson (1982) presented mathematical models for the genetic determination of variation among individuals in a population using RFLP.

Use of RFLPs to measure genetic relatedness among strains and closely related species of plant pathogenic fungi is still in its beginning. Genetic variability of several plant pathogenic fungi, *Armillaria mellea* (Anderson et al. 1987), *Sclerotinia* species (Kohn et al. 1988), *Septoria tritici* (McDonald and Martinez 1990) and *Aspergillus* species (Someren et al. 1991) has been studied using RFLP genetic markers. In *C. gloeosporioides* two population subgroups were recognized by distinct RFLP patterns detected by human minisatelite probes for hypervariable regions within the genome (Braithwaite and Manners 1989). Linkage maps have been developed for the lettuce downey mildew fungus, *Bremia lactucae* using RFLPs as genetic markers (Hulbert and Michelmore 1988; Hulbert et al. 1988). Hulbert et al. (1988) also reported the linkage of an avirulance gene and a RFLP locus and suggested the possibility of cloning the avirulance gene by chromosome walking.

Castle et al. (1987) distinguished the commercial mushroom, *Agaricus brunnescens*, from *A. bitorquis* using distinct RFLP patterns. These RFLP patterns were used in the identification of homokaryotic, heterokaryotic and hybrid strains of this fungus (Castle et al. 1987) . Summerbell et al. (1989) followed the segregation of RFLPs in wild collected and artificially synthesized heterokaryotic strains of *A. brunnescens* to investigate meiosis and the meiotic recombination in this fungus.

The objective of this study is to determine if type 1 and type 2 *Colletotrichum gloeosporioides* are genetically distinguishable. This will be done by examining rDNA polymorphisms, the diversity of cutinase enzymes at the isozyme and molecular level, and other molecular markers to examine RFLPs in type 1 and type 2 strains of *C. gloeosporioides* causing PFD of Tahiti lime and sweet orange.

Materials and Methods

Strains of *Colletotrichum gloeosporioides*

Strains of *C. gloeosporioides,* host and geographic location are listed in the appendix A. Each strain is a single spore culture grown. The place, year, and the host tissue of isolation are mentioned in appendix A.

DNA Extraction

Fungal mycelium was grown in potato dextrose broth (PDB) for seven days, harvested, frozen at -80 °C overnight and lyophilized to complete dryness. The mycelium was ground into a powder in liquid nitrogen using a mortar and pestle. The mycelium powder was mixed with extraction buffer (100 mM Tris pH 8.0, 50 mM EDTA, 100 mM NaCl, 10 mM ß-mercaptoethanol, 1% SDS, in H_2O) to make a slurry and incubated at 65 °C for 30 min. One half volume of 5 M potassium acetate (60 ml of 5 M potassium acetate, 11.5 ml glacial acetic acid and 28.5 ml H_2O) was added to samples and incubated on ice for 30 min. The supernatant was collected by centrifugation at 12000 x g for 15 min and was treated with 30-50 μg/ml of DNase free RNase (30 min at 37 °C, Sigma Chemical Co., St. Louis, MO). After RNase treatment 200-250 μg/ml Proteinase K (Sigma Chemical Co., St. Louis, MO) was added and incubated for an additional 20 min. Samples were purified by phenol:isoamyl alcohol:chloroform (25:1:24 by volume) extraction and DNA was precipitated by addition of a two-fold volume of absolute ethanol. DNA pellets were dissolved in 100 μl of TE (10 mM Tris pH 8.0, 1 mM EDTA) and further purified by precipitation with 0.7 volume of PEG/NaCl ((20% PEG 8000 in 2.5 M NaCl, Sigma Chemical Co., St. Louis, MO) for 20-30 min on ice. Precipitated DNAs were resuspended in TE and stored at -20 °C.

DNA Cloning and Restriction Enzyme Mapping

The rDNA of *C. gloeosporioides* was identified by
heterologous hybridization with *N. crassa* rDNA unit repeat
(pMF2, Free et al. 1979). Total DNA from *C. gloeosporioides*
strains H-25B and H-48 was digested with restriction enzyme
*Pst*I and fractionated on a 0.7% agarose (FMC BioProducts,
Rockland, ME) gel. The piece of the gel containing the 7 to
10 kb (H-25B) or 6-10 kb (H-48) DNA range was cut out and
the DNA eluted by the freeze squeeze method (Thuring et al.
1975). The DNA was ligated to *Pst*I-cut pUC119 (Sambrook et
al. 1989; Yanisch-Perron et al. 1985) and transformed into
Escherichia coli strain ER1647 (*E. coli* K-12 *mcrB*⁻, Ref.
Raleigh et al. 1989; Woodcock et al. 1989) or DH5-α
(Sambrook et al. 1989). Ligation, preparation of competent
cells and transformation was carried out according to
Sambrook et al. (1989). Clones hybridizing to pMF2 were
identified and restriction mapped. For constructing
restriction maps, single and double restriction enzyme
digestions of two presumptive rDNA clones, called pCGR1 (8.4
kb from strain H-25B), pCGR2 (6.8 kb from strain H-48), were
size fractionated in 1% agarose gels. Regions homologous to
the LSU and SSU rRNA of *N. crassa* were mapped by Southern
hybridization to heterologous probes subcloned from plasmid
pMF2 (Free et al. 1979; Martin 1990). The probe specific to
LSU was a 1.7 kb *Xba*I + *Bam*HI fragment comprising all but

150 bp of the 5' end of the 17S coding region. A 2.9 kb
EcoRI fragment containing all but approximately 700 bp of
the 3' end of the 26S coding region in addition to 200 bp of
transcribed spacer sequences adjacent to 5' end was used as
the probe specific to LSU. These subclones were provided by
Dr. F. N. Martin, Plant Pathology Department, University of
Florida. A probe to detect the 5.8 rRNA gene was prepared by
polymerase chain reaction using primer flanking the gene.
The primers 5' TCCGTAGGTGAACCTGCGC 3' and 5'
GCTGCGTTCTTCATCGATGC 3' amplify a 290 bp fragment which
includes the transcribed spacer of the 3' end of the SSU
rRNA gene and the entire 5.8S gene (White et al. 1990).

Enzyme Assays and Electrophoresis of Cutinase

Colletotrichum gloeosporioides cultures were grown in a
mineral medium (Hankin and Kolattukudy 1968) amended with
tritiated cutin as the sole carbon source. Esterase
activities of strains were measured using *p*-nitrophenyl
butyrate (PNB) and *p*-nitrophenyl palmitate (PNP) as model
substrates (Köller and Kolattukudy 1982; Köller and Parker
1989; Purdy and Kolattukudy 1975a). Assays for cutinase
activity, sodium dodecyl sulfate polyacrylamide gel
electrophoresis (SDS-PAGE) and detection of active serine
esterases by tritium-labelled diisopropyl fluorophosphate
(^{3}H-DFP) were as previously described (Köller and Parker
1989; Trail and Köller 1990). These experiments were

conducted by Dr. Wolfram Köller at the New York State

Agricultural Experiment Station, Geneva.

Probes Containing Cutinase Gene Sequences

Oligonucleotide primers, 5' TGCCCCAAGGTCATCTACATC 3'and

5'GAAGTTGGAGGCCAGGTCGGC 3' were synthesized to amplify a 220

bp fragment (intron and flanking sequences) of *C.*

gloeosporioides cutinase gene by polymerase chain reaction

(PCR). The PCR reaction mixture was prepared in a total of

100 μl containing 100 pM of each primer, 1.25 mM each of

dATP, dTTP, dCTP and dGTP, 2 μg of template DNA, and 10 μl

of reaction buffer (50mM KCl, 10 mM Tris-HCl pH 8.3, 1.5mM

$MgCl_2$, 0.01% gelatin). The PCR mixture was denatured by

boiling 10 min and chilled on ice before adding 2 units of

Taq DNA Polymerase (Promega Inc. Madison, WI). The PCR

temperature cycles were programmed as following in a Coy

Temp Cycler II (Coy Corp., Grass Lake, MI). Denaturation

temperature was 94 °C, annealing temperature was 37 °C and

primer extension was at 72 °C. The first cycle was run 6 min

at 94°C, 2 min at 37 °C and 3 min at 72°C and the subsequent

30 cycles were run at 1, 2, and 3 min time intervals,

respectively, at these temperatures. Primer extension time

for the final cycle was 10 min. The fragments amplified by

PCR were labelled with [32]P dCTP or digoxigenin (dig) dUTP

(Boehringer Mannheim Corp. Indianapolis, IN; see Appendix

C). Both a 220 and a 260 bp DNA fragment amplified by PCR

from strain H-48 hybridized to a genomic clone containing,
the cutinase gene, a 2.2 kb *Sph*I DNA fragment, from *C.
gloeosporioides* (Ettinger et al. 1987). This clone was
provided by Dr. M. B. Dickman, Department of Plant
Pathology, University of Nebraska, Lincoln, NE.

High stringency Southern hybridization (Southern 1975)
using ^{32}P-labelled probes was carried out according to
methods described by Sambrook et al. (1989). Hybridization
and washing of blots were carried out at 68 °C. First and
second washes were with 2X SSC, 0.1% SDS and 0.1X SSC, 0.1%
SDS respectively. The conditions of low stringency Southern
hybridization were as follows. DNA hybridization was at
65 °C and first and second washes were with 2X SSC at 55 °C.
Autoradiography was performed with Kodak X-OMAT AR5 film
(Eastman Kodak Co., Rochester, NY) and Dupont Hi-Plus
intensifying screens at -80 °C.

Detection of Restriction Fragment Length Polymorphisms

Plasmids containing *N. crassa* genes for anthranilate
synthetase (pNC2, Schechtman and Yanofsky 1983), glutamate
dehydrogenase (pJR2, Kinsey and Rambosek 1984), histidinol
dehydrogenase (pNH60, Legerton and Yanofsky 1985), and ß-
tubulin (pSV50, Vollmer and Yanofsky 1986) were used to
detect DNA polymorphisms. Clones of *N. crassa* genes were
obtained from the Fungal Genetics Stock Center (Department
of Microbiology, University of Kansas Medical Center, Kansas

City, KS). Southern hybridization of ^{32}P-labelled probes were carried out according to methods described by Sambrook et al. (1989) and Appendix C.

Results

Ribosomal DNA is Polymorphic in *Colletotrichum gloeosporioides*

Southern hybridization of ^{32}P labelled pMF2 to *Pst*I-digested total blots from *C. gloeosporioides* strains detected polymorphic forms of rDNA (Figure 3.1 and 3.2). The type 1 strains (see Chapter 2 and Appendix A) contained only a single form (8.4 kb *Pst*I fragment) of rDNA (Figures 3.1 and 3.2). Although the size of the rDNA fragment in type 1 strains H-36 and OCO appears to be slightly higher than 8.4 kb in Figure 3.1, other restriction enzyme digestion tests concluded that it is 8.4 kb in size (compare figures 3.5, 3.6, and 3.7). The 8.4 kb *Pst*I rDNA fragment was cloned from Type 1 strain, H-25B and will be referred to as type 1 rDNA. The restriction fragments obtained by digesting with 10 restriction enzymes and the restriction map of cloned type 1 rDNA unit (pCGR1) for these enzymes are illustrated in Table 3.1 and Figure 3.3 respectively. The restriction map of the cloned rDNA unit was compared with the total rDNA restriction fragments of the strain H-25B for 7 enzymes and was identical (Figure 3.4). The map of cloned rDNA unit from H-25B was tested against all type 1 strains for three

restriction enzymes, *Hind*III, *Sph*I and *Sst*I (Figures 3.5,
3.6, and 3.7 respectively). All the type 1 strains fell into
an identical group and the hybridization fragments for the
three enzymes agreed with restriction map of the cloned rDNA
fragment from Strain H-25B. Several subcloned fragments from
the NTS region were tested to determine if they can
specifically hybridize to type 1 strains. A 0.4 kb *Kpn*I-*Pst*I
subclone (pCGR1N) from the 3' end of NTS region (Figure 3.3)
was found to hybridize only to type 1 rDNA. The same total
DNA blot in Figure 3.1 was reprobed with the type 1-specific
subclone after removing the previous probe and only type 1
strains show hybridization to the subclone (compare Figures
3.1 and 3.8).

Ribosomal DNA among type 2 strains (see chapter 2) was
polymorphic for *Pst*I (Figures 3.1 and 3.2), *Sph*I (Figure
3.6), and *Sst*I (Figure 3.7). However, *Hind*III digested DNA
shows a similar pattern of rDNA polymorphism among all type
2 strains (Figure 3.5). Two hybridizing *Pst*I fragments were
detected in strains H-48, 180269, 226802 (8.4 and 6.8 kb),
H-11 (5.0 and 3.4 kb), and H-47 (8.4 and 7.8 kb) by ^{32}P
labelled pMF2 (Figures 3.1 and 3.2). However, the
hybridization intensity of the 8.4 kb band in strains 180269
and 226802 was very low and almost undetectable compared to
6.8 kb hybridizing band (Figure 3.1). All other type 2
strains had only one 6.8 kb hybridizing band. The 6.8 kb
*Pst*I fragment was cloned from strain H-48, and the

restriction map was identical when compared with the total rDNA restriction fragments of the strain H-48 for 7 enzymes (Figure 3.4). The cloned 6.8 kb *Pst*I fragment from type 2 strain H-48 hybridizing to pMF2 will be referred to as pCGR2 or type 2 rDNA. The restriction fragments obtained by digestion of clones of type 1 and type 2 rDNA unit with various restriction enzymes and restriction enzyme maps are listed in Table 3.1. The length of type 1 rDNA differs from type 2 by the size of the NTS region of the unit (Figure 3.3). The restriction map of pCGR2 is distinct from that of type 1 rDNA and the NTS is 1.6 kb shorter.

Ribosomal RNA Genes

In addition to the length heterogeneity, type 1 and type 2 rDNA units differ by having restriction site polymorphisms and addition and deletion of restriction sites within coding regions for rRNA as well as intergenic regions. Restriction sites for *Sma*I and *Sst*I within the SSU coding region and a *Eco*RI site within the 5.8 S coding region were found in type 1 rDNA. For the 10 restriction enzyme sites examined none was detected within the SSU rRNA and 5.8S RNA coding regions of type 2 rDNA. The coding region for the LSU lies within *Bam*HI and *Eco*RI sites for both type 1 and type 2 rDNA, and were detected as 3.1 and 3.0 kb hybridizing fragments respectively. Restriction site

TABLE 3.1 Restriction fragments obtained by complete digestion of the three ribosomal DNA clones

No.	Restriction enzyme	Fragment size (kb)	
		pCGR1	pCGR2
1	*Pst*I	8.4	6.8
2	*Hind*III	4.1	3.2
		3.2	3.1
		1.1	0.5
3	*Sph*I	6.0	4.2
		1.4	2.5
		1.0	0.1
4	*Eco*RI	2.7	3.2
		2.5	2.6
		2.4	1.0
		0.8	
5	*Bam*HI	5.5	4.0
		2.9	2.8
6	*Kpn*I	6.2	6.4
		0.6	0.4
		0.6	
		0.6	
		0.4	
7	*Hinc*II	3.3	3.0
		2.8	1.6
		1.4	1.0
		0.7	0.6
		0.2	0.6
8	*Xba*I	7.8	6.3
		0.6	0.5
9	*Sma*I	2.2	4.3
		2.2	1.6
		2.0	0.9
		1.0	
		1.0	
10	*Sst*I	4.6	4.6
		2.0	1.8
		1.8	0.4
11	*Cla*I	N	N

N = No restriction site detected

polymorphisms for enzymes *Hind*III, *Eco*RI, *Sst*I, *Hinc*II, and *Sma*I were detected within the coding region for LSU rRNA in the type 1 and type 2 rDNA forms. The NTS region of type 1 rDNA has 4 *Kpn*I sites with three present at equal distance of 0.6 kb, whereas within the NTS region of type 2 rDNA there is only a single *Kpn*I site. The additional 1.6 kb NTS region fragment in type 1 rDNA contains two *Kpn*I sites, each 0.6 kb apart and a 0.4 kb *Pst*I/*Kpn*I type 1 rDNA specific fragment.

Diverse Cutinases and Cutinase Genes are Found in Type 1 and Type 2 Strains of *Colletotrichum gloeosporioides*

Cutinase production by fungal mycelium can be induced by cutin monomers (Lin and Kolattukudy 1978). Similarly, all Tahiti lime and Sweet orange strains of *C. gloeosporioides* excreted esterases under these inductive conditions when cutin was used as the sole carbon source. Although, both model cutin substrates, *p*-nitrophenol-butyrate and -palmitate, were hydrolyzed, the ratio of these two activities was remarkably different (Table 3.2). Cutinolytic activity was identified for all isolates and was consistently higher at pH 6.0 than at the alkaline pH of 9.5. Extracellular proteins were labelled with ^{3}H-DFP and used as active site probe for serine esterase. Two esterases in the molecular weight range common to many known fungal cutinases (17kd-32kd, Kolattukudy 1980; Tanabe et al 1988;

TABLE 3.2 Extracellular enzyme activities of *Colletotrichum gloeosporioides*

Strain	PNBase mg/ml	PNPase mg/ml	Cutinase kBq/h/mg pH 6	pH9.5	ratio	PNB/PNP	PNB/ Cutinase pH 6
H-1	4731	255	18.7	2.0	9.4	18.6	254
IMB-3	6475	351	25.4	2.8	9.1	18.5	255
H-3	3678	302	23.9	4.5	5.3	12.2	154
H-4	8784	752	57.5	8.0	7.2	11.7	153
LP-1	5844	499	28.6	8.3	3.4	11.7	204
H-46	477	50	5.9	1.5	3.9	9.6	81
H-12	4692	554	40.0	1.3	30.8	8.5	117
H-25B	3627	488	27.5	5.5	5.0	7.4	132
Maran	3575	484	23.1	10.3	2.2	7.4	155
H-9	2193	329	18.8	4.2	4.4	6.7	117
H-48	4099	662	27.0	6.1	4.4	6.2	152
180269	4668	771	28.1	4.9	5.7	6.1	166
226802	4695	782	49.3	10.7	4.6	6.0	95
H-36	1863	369	23.7	5.8	4.1	5.0	78
Control	1456	332	12.8	7.9	1.6	4.4	144

PNB=*p*-nitrophenyl butyrate
PNP=*p*-nitrophenyl palmitate
Control consisted of all treatment without a fungal strain.

Trail and Köller 1990) were present for all strains (Figure
3.9). The molecular weight of these proteins differed among
strains and was correlated to *C. gloeosporioides* RFLP-types.
All strains of type 1 contained bands of 24 and 21 kd,
whereas all strains of type 2 contained 26 and 19 kd bands.
An additional esterase with a molecular weight of about 70
kd, which was not reported for the papaya isolate of *C.
gloeosporioides* (Dickman et al. 1982), was present
throughout the set of isolates. The high molecular weight
esterase was slightly larger for type 1 strains. The
relative contribution of this high molecular weight esterase
to the total esterase and cutinase activities remains
unknown. The enzyme might be similar to the 60 kd alkaline
cutinolytic esterase isolated from *C. lagenarium* (Bonnen and
Hammerschmidt 1989a) or the 54 kd non-specific esterase of
N. haematococca (Purdy and Kolattukudy 1975a).

A genomic clone containing the cutinase gene from a
papaya strain of *C. gloeosporioides* (Ettinger et al 1987)
was ^{32}P-labelled and used to probe *Sph*I-digested DNA of *C.
gloeosporioides* from citrus. This clone contained a 2.2 kb
*Sph*I fragment which included the cutinase gene (189 bp exon-
52 bp intron-486 bp exon) and 5' and 3' flanking sequences
(Ettinger et al 1987). The probe hybridized to a 2.2 kb *Sph*I
fragment only in type 2 strains (Figure 3.10). DNA from type
1 strains showed no detectable level of hybridization at the
high level of stringency (see materials and methods) used

for Southern hybridization. Although the cutinase gene sequence shows no polymorphism among type 2 strains for the restriction enzyme *Sph*I, a restriction fragment length polymorphism can be detected among type 2 strains for *Hind*III (Figure 3.11). A 9.0 kb *Hind*III fragment hybridized to the probe in all type 2 strains except 226802 which show hybridization to a 8.0 kb fragment. Type 1 strains did not show any detectable level of hybridization to the probe at this level of stringency used for Southern hybridization (see Appendix C). The long exposure (>1 month) of this blot resulted in appearance of 4.8, 5.4, 6.6, 7.4, and 9.0 kb *Hind*III hybridizing fragments of low level homology (Figure 3.12). Non-radioactive hybridization (Genius, Boehringer Mannheim Corp. Indianapolis, IN) under low stringency conditions shows weak hybridization of the probe to a 7.4 kb *Hind*III fragment from type 1 strains (Figure 3.13).

Polymerase chain reaction amplification using oligonucleotide primers flanking an intron sequence in the cutinase gene resulted in a single 220 bp fragment when H-3 (type 1 strain) or H-12 (type 2 strain) DNA was used as a template. However, strains LP-1 (type 1) and H-48 (type 2) produced two amplified fragments, 220 and 260 bp (Figure 3.14). The 220 and 260 bp fragment amplified by PCR from strain LP-1, when used as probes for Southern hybridization, also hybridized to numerous *Hind*III fragments. Approximately 5-10 restriction fragments, ranging in size from 0.5 to >10

kb were identified (Figure 3.15 and 3.16 respectively).
Restriction fragments from all type 1 strains were almost
entirely identical. Hybridizing fragments from the type 2
strains showed dissimilar patterns. Hybridization of these
two probes to total DNA resulted in distinct DNA fingerprint
for type 1 strains.

Subgroups of *Colletotrichum gloeosporioides* have Distinct RFLP Patterns Detected by Many Genetic Markers

Four clones of *N. crassa* genes were used as
heterologous probes to identify additional genetic loci in
*Hind*III-digested DNA from *C. gloeosporioides* strains. The
probe pSV50, containing the gene for ß-tubulin, hybridized
to a 3.2 kb fragment in type 1 strains but a 5.0 kb fragment
in type 2 strains (Figure 3.17). The probe, pJR2, containing
the gene for glutamate dehydrogenase, hybridized to a 3.2 kb
fragment only in type 2 strains, but only diffuse
hybridization was observed in type 1 strains (Figure 3.18).
The probe, pNH60, containing the gene for histidinol
dehydrogenase, hybridized to both a 3.8 and a 4.3 kb
fragment in type 1 strains but hybridized to 3.3 and 4.8 kb
fragments in type 2 strains (Figure 3.19).
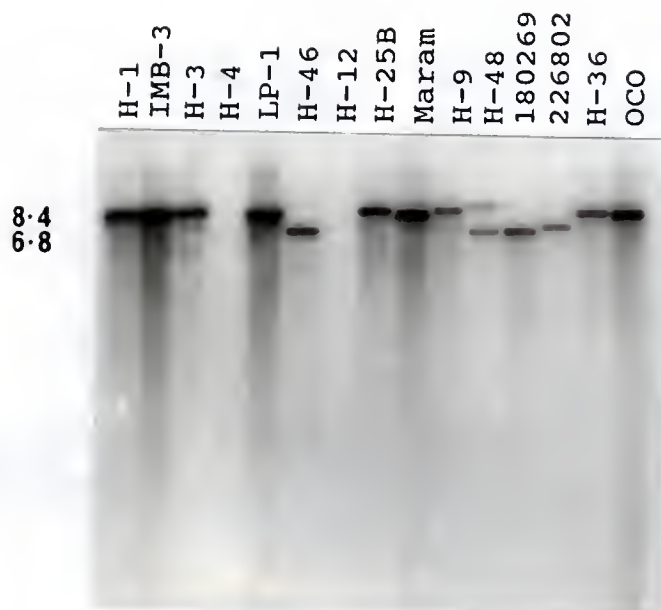
Figure 3.1 Polymorphic forms of ribosomal DNA unit in *C. gloeosporioides* strains. Total DNA was digested with *Pst*I and Southern hybridized with ^{32}P labelled pMF2 (*N. crassa* rDNA unit repeat). Lanes H-12, 226802, H-36, and OCO shows slower migration of DNA than expected. Lane H-4 DNA is degraded. Numbers at the left indicate the size of restriction fragments in kilobases (kb).
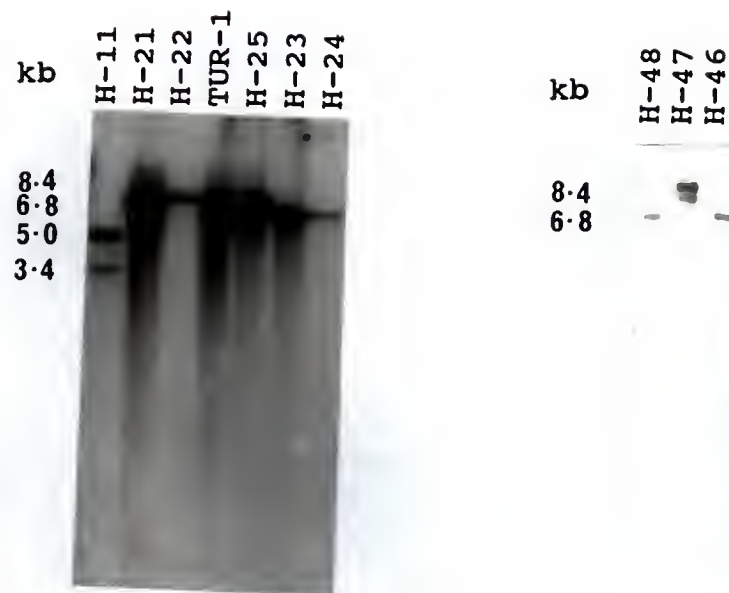
Figure 3.2 Various restriction fragments contain ribosomal
DNA in type 2 strains of *C. gloeosporioides*.
Total DNA was digested with *Pst*I and Southern
hybridized with ^{32}P labelled pMF2 (*N. crassa* rDNA
unit repeat). Numbers at the left indicate the
size of the major restriction fragments in
kilobases (kb).
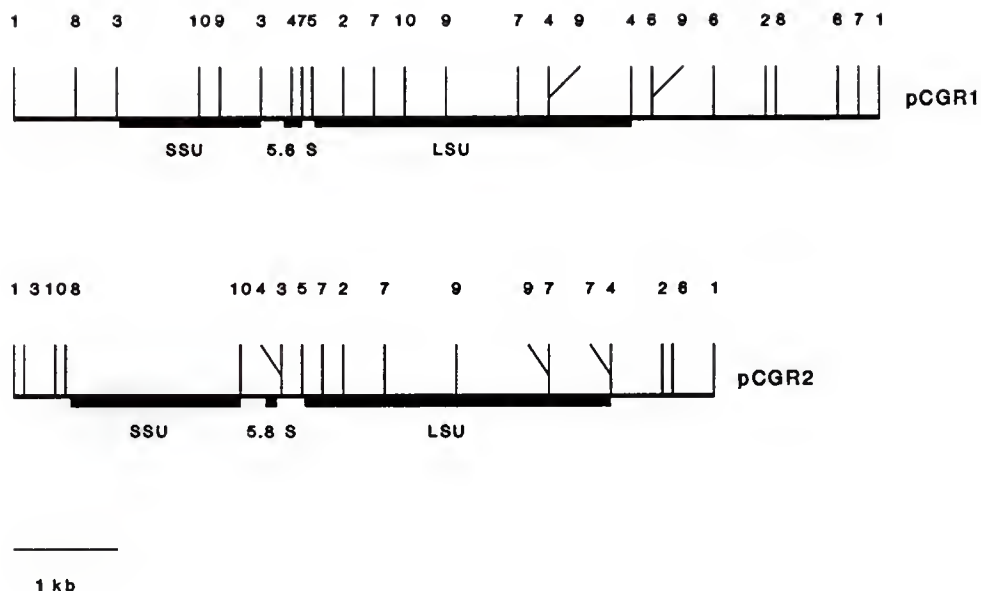
Figure 3.3 Restriction enzyme maps for cloned rDNA units (pCGR1 from *Colletotrichum gloeosporioides* type 1 strain, H-25B, and pCGR2 from type 2 strain, H-48). Regions hybridizing to large subunit rRNA (LSU), small subunit rRNA (SSU) from *N.crassa* and the PCR amplified 5.8S rRNA gene from *C. gloeosporioides* are indicated by solid boxes. Restriction enzyme sites are as follows. *Pst*I (1), *Hind*III (2), *Sph*I (3), *Eco*RI (4), *Bam*HI (5), *Kpn*I (6), *Hinc*II (7), *Xba*I (8), *Sma*I (9), and *Sst*I (10).
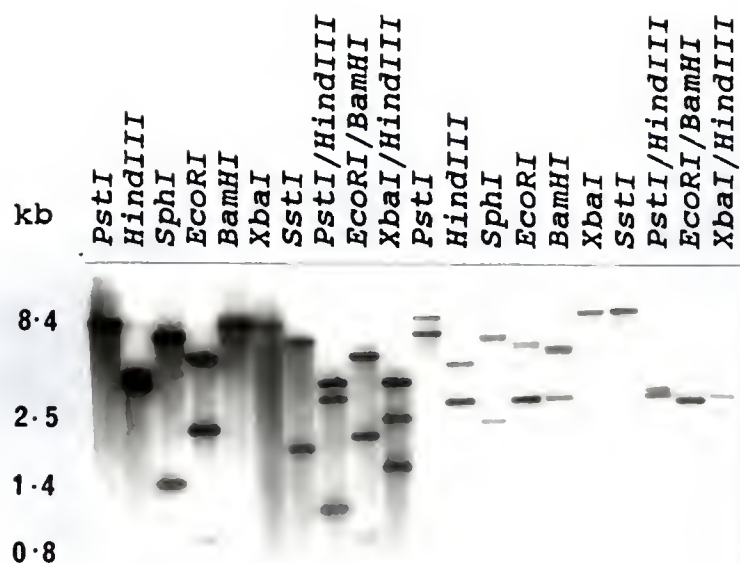
Figure 3.4 The rDNA of *C. gloeosporioides* strains H-25B
(first 10 lanes from left) and H-48 (next 10
lanes) digested with various restriction enzymes
and detected by Southern hybridization using [32]P
labelled pMF2 (*N. crassa* rDNA unit repeat) as a
probe. The numbers at the left indicate the size
of major restriction fragments in kilobases (kb).
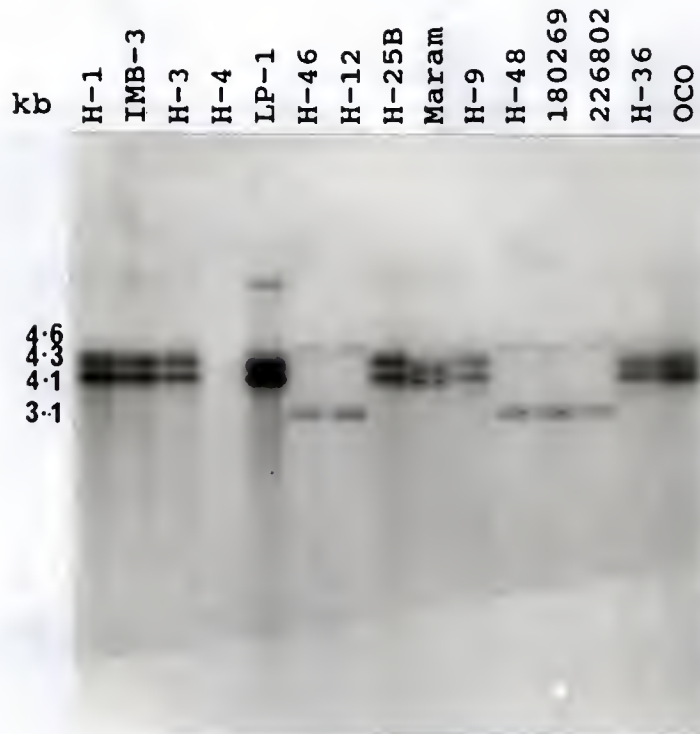
Figure 3.5 Ribosomal DNA polymorphism in *C. gloeosporioides* strains. Total DNA was digested with *Hind*III and Southern hybridized with ^{32}P labelled pMF2 (*N. crassa* rDNA unit repeat). H-4 DNA is degraded. The numbers at the left indicate the size of restriction fragments in kilobases (kb).
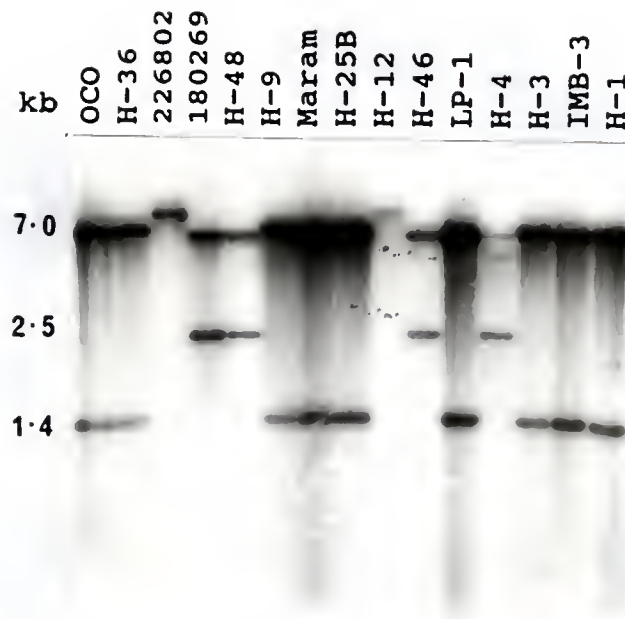
Figure 3.6 Ribosomal DNA polymorphism in *C. gloeosporioides* strains. Total DNA was digested with *Sph*I and Southern hybridized with ^{32}P labelled pMF2 (*N. crassa* rDNA unit repeat). The numbers at the left indicate the size of major restriction fragments in kilobases (kb).
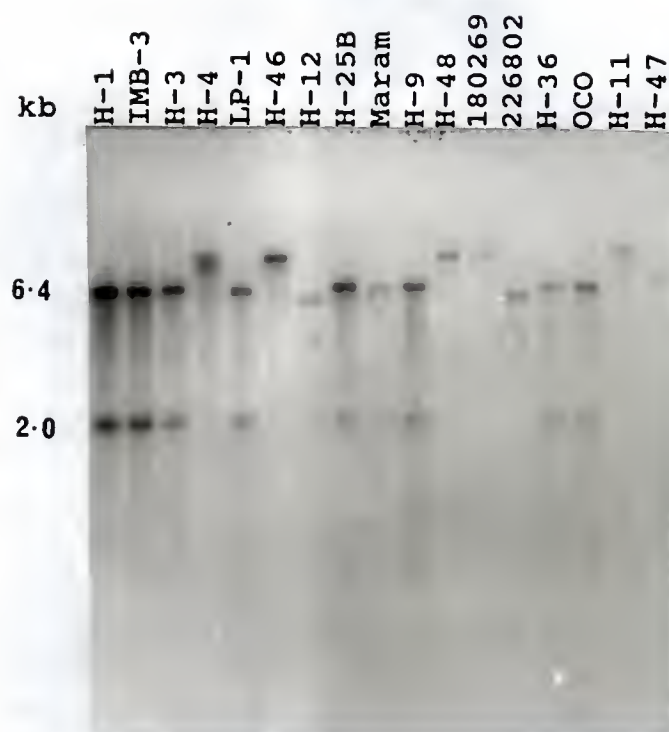
Figure 3.7 Ribosomal DNA polymorphism in *C. gloeosporioides* strains. Total DNA was digested with *Sst*I and Southern hybridized with ^{32}P labelled pMF2 (*N. crassa* rDNA unit repeat). The numbers at the left indicate the size of major restriction fragments in kilobases (kb).
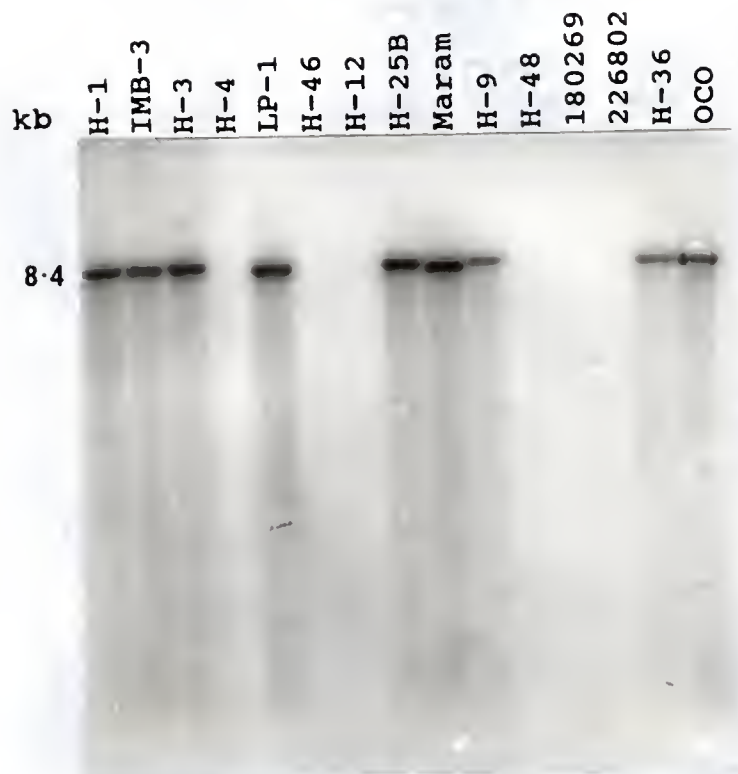
Figure 3.8 A 0.4 kb *PstI/KpnI* fragment (pCGR1N) from the
non-transcribed spacer region of cloned rDNA
unit from *C. gloeosporioides* isolate H-25B
hybridizes only to the 8.4 kilobase (kb) rDNA
form in type 1 strains. Total DNA was digested
with *PstI* and Southern hybridized with [32]P
labelled pCGR1N.
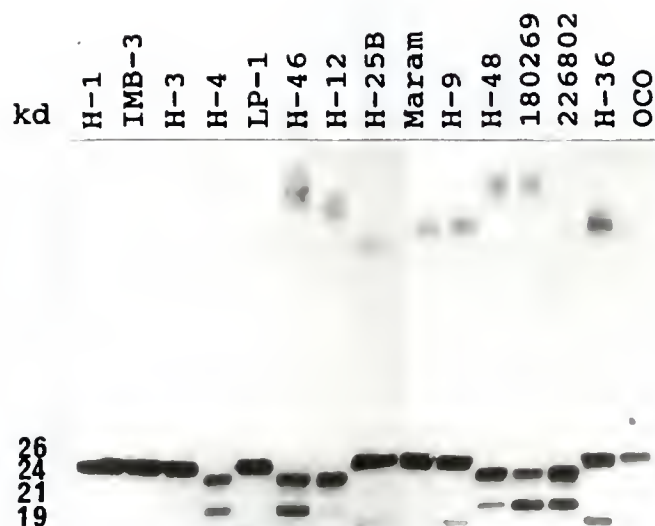
Figure 3.9 Fluorography of [3]HDFP-treated proteins after SDS-polyacrylamide gel electrophoresis of extracellular fluid from *C. gloeosporioides* cultures grown on cutin as the sole carbon source. Numbers at the left indicate the molecular weight in kilodaltons (kd).
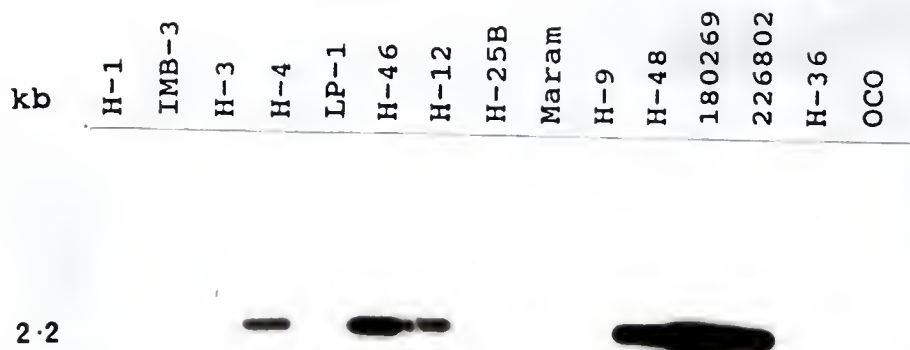
Figure 3.10 Cutinase gene in *C. gloeosporioides* type 2
strains. Total DNA was digested with *Sph*I and
hybridized to a ^{32}P labelled probe containing a
cloned cutinase gene. Numbers at the left
indicate the size of restriction fragment in
kilobases (kb).
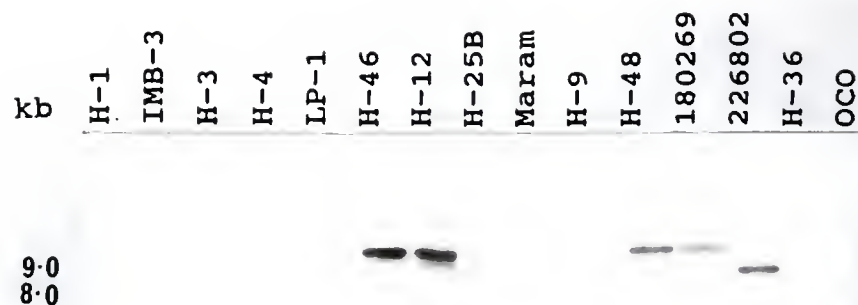
Figure 3.11 Restriction fragments hybridize to the cloned cutinase gene only within type 2 strains. Total DNA was digested with *Hind*III and hybridized to a ^{32}P labelled probe containing a cloned cutinase gene sequence from *C. gloeosporioides*. H-4 DNA is degraded. Numbers at the left indicate the size of restriction fragments in kilobases (kb).

65
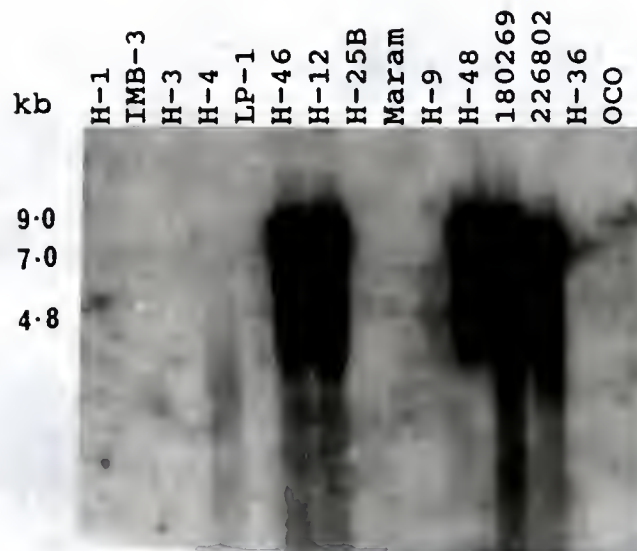
Figure 3.12 Hybridization of total DNA from the indicated
strains to a cloned cutinase gene sequence. The
DNA was digested with HindIII and hybridized to
a ³²P labelled probe containing a cloned
cutinase gene sequence from *C. gloeosporioides*.
The blot was overexposed by placing it next to
X-ray film for more than 4 weeks. H-4 DNA is
partially degraded. Numbers at the left indicate
the size of major restriction fragments in
kilobases (kb).
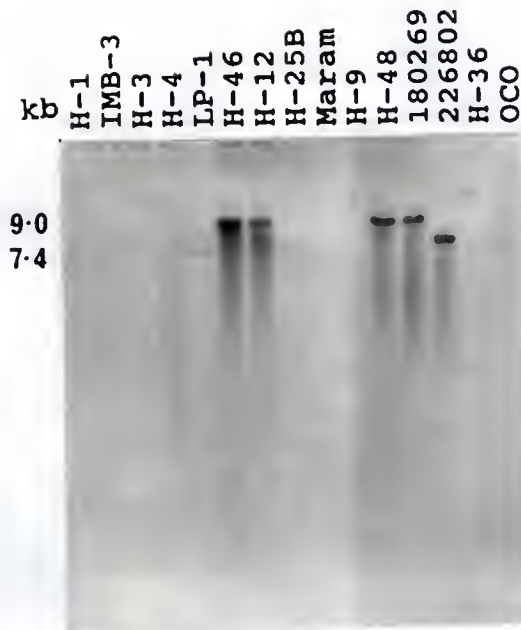
Figure 3.13 Presumptive cutinase genes in *C. gloeosporioides* type 1 and type 2 strains. Total DNA was digested with *Hind*III and Southern hybridized to a digoxigenin labelled probe containing a cloned cutinase gene sequence from *C. gloeosporioides*. H-4 DNA is degraded. Numbers at the left indicate the size of restriction fragments in kilobases (kb).
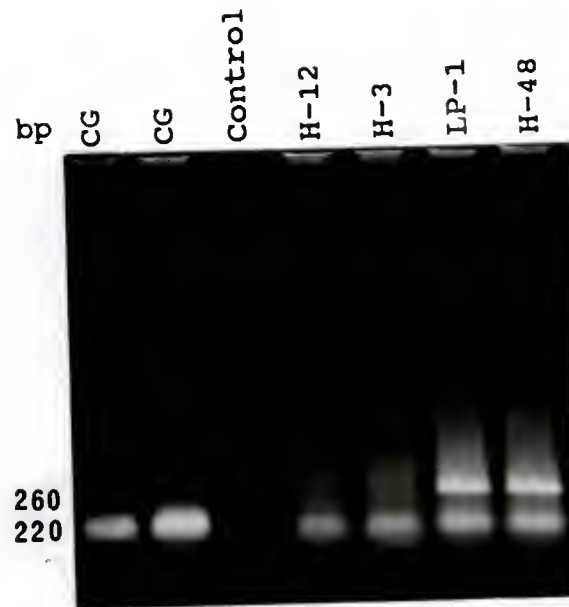
Figure 3.14 PCR Amplified fragments using oligonucleotide
primers flanking the intron in the
C. gloeosporioides cutinase gene. Lanes contain
DNA from PCR reactions using for the template
the cloned cutinase gene (CG), or total DNA from
the strains indicated. The control reaction lane
contained no template DNA. Numbers at the left
indicate the size of DNA restriction fragments
in basepairs (bp).
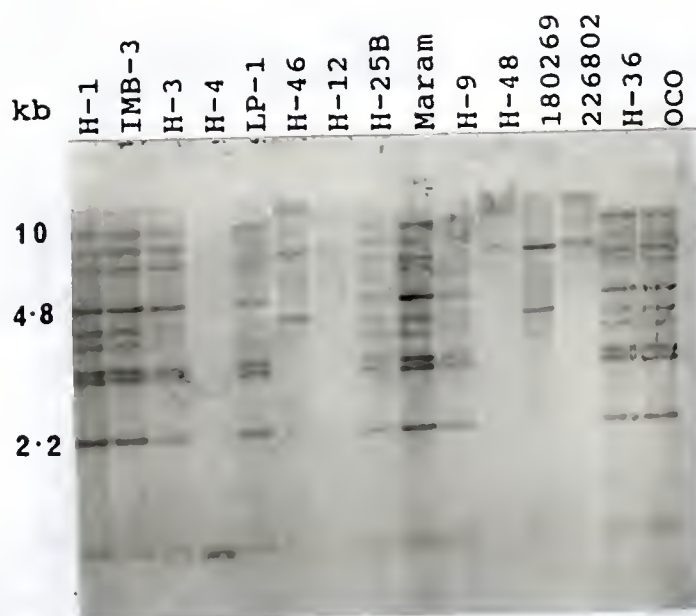
Figure 3.15 RFLP patterns in total DNA from the indicated *C. gloeosporioides* strains detected by Southern hybridization to a 220 bp fragment amplified by PCR. The total DNAs were digested with *Hind*III and the probe was labelled with digoxigenin dUTP. H-4 DNA was partially degraded. Numbers at the left indicate the size of major restriction fragments in kilobases (kb).
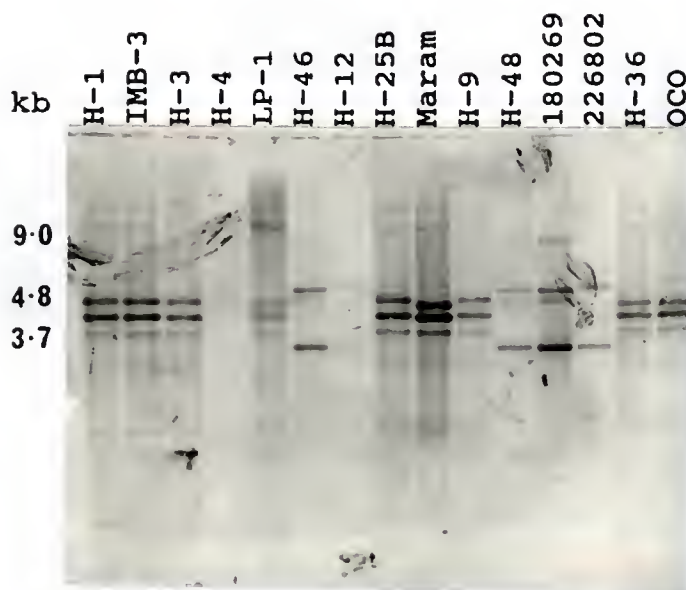
Figure 3.16 RFLP patterns in total DNA from the indicated *C.
gloeosporioides* strains detected by Southern
hybridization to a 260 bp fragment amplified by
PCR. The total DNAs were digested with *Hind*III
and the probe was labelled with digoxigenin
dUTP. H-4 DNA was partially degraded. Numbers at
the left indicate the size of major restriction
fragments in kilobases (kb).

Figure 3.17 RFLP patterns in total DNA from the indicated *C. gloeosporioides* strains detected by Southern hybridization to the ^{32}P labelled ß-tubulin gene from *N. crassa* (plasmid pSV50). The total DNAs were digested with *Hind*III. Numbers at the left indicate the size of restriction fragments in kilobases (kb).
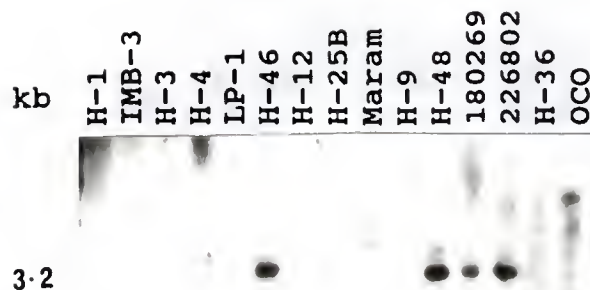
Figure 3.18 RFLP patterns in total DNA from the indicated *C. gloeosporioides* strains detected by Southern hybridization to the ^{32}P labelled glutamate dehydrogenase gene from *N. crassa* (plasmid pJR2). The total DNAs were digested with *Hind*III. Numbers at the left indicate the size of the major restriction fragment in kilobases (kb).
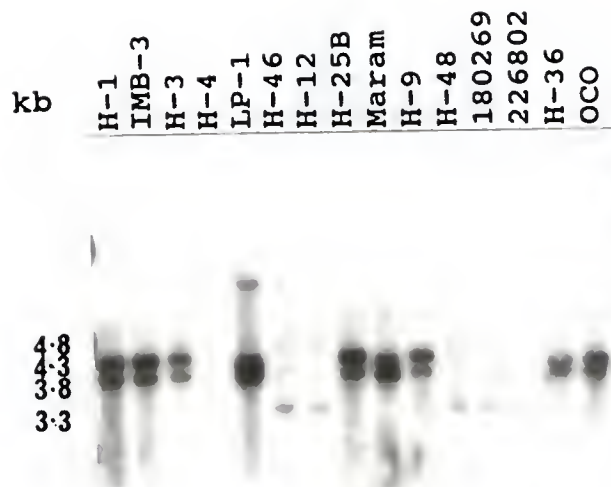
Figure 3.19 RFLP patterns in total DNA from the indicated *C. gloeosporioides* strains detected by Southern hybridization to the ^{32}P labelled histidinol dehydrogenase gene from *N. crassa* (plasmid pNH60). The total DNAs were digested with *Hind*III. Numbers at the left indicate the size of the major restriction fragments in kilobases (kb).

Discussion

In Chapter 2, two types of *Colletotrichum gloeosporioides* strains were described based on morphological and growth characteristics. However, morphological features vary with culture conditions and time. Morphological variability always has been the case with *C. gloeosporioides* (Burger 1921; Arx 1957). Despite confusion associated with morphological inconsistency, at the molecular level we see distinct differences between type 1 and type 2 strains.

The rDNA unit proved to be a molecular marker which can be used to detect type 1 strains of *C. gloeosporioides* (Figure 3.8). Based on the Southern hybridization results, morphologically stable type 1 strains contain a single form of rDNA. The restriction map of the cloned rDNA unit (Figure 3.3, pCGR1) from type 1 strain, H-25B was identical when compared with the restriction map of the genomic rDNA (compare Figures 3.3 and 3.4). The three enzyme sites *Hind*III, *Sph*I, and *Sst*I examined for all type 1 strains agree with the map of the rDNA clone from strain H-25B indicating that the form of rDNA present in type 1 strains has been cloned and mapped (compare figures 3.3, 3.4, 3.5, 3.6 and 3.7). The size of the type 1 rDNA unit, 8.4 kb in *C. gloeosporioides*, is within the range of observed rDNA unit repeat sizes for filamentous fungi such as *N. crassa*, 9.23 kb (Free et al. 1979), *Aspergillus nidulans*, 7.8 kb (Borsuk

et al. 1982), *Schizophyllum commune* 9.2-9.6 kb (Specht et al. 1984), and *Thanatephorus praticola*, 8.8 kb (Vilgalys and Gonzalez 1990). The given order of rRNA genes, 5' SSU-5.8S-LSU 3' (Figure 3.3) within the rDNA unit repeat is similar in all the fungi examined (Free et al. 1979; Cihlar and Sypherd 1980; Borsuk et al. 1982; Cassidy et al. 1984; Buckner et al 1988; Garber et al. 1988; Vilgalys and Gonzalez 1990). The rDNA unit repeats in *C. gloeosporioides* may also code for large, 35-37S, precursor rRNAs which give rise to SSU, 5.8S, and LSU rRNAs required for the building of 80S ribosomes (Russell et al. 1976; Bell et al. 1977; Planta et al. 1980). The search for a specific fragment of rDNA which can detect only type 1 rDNA was successful. The sub-clone pCGR1N containing a 0.4 kb *PstI-KpnI* from the NTS region was strain specific (compare Figures 3.1 and 3.8). Therefore, type 1 strains can be defined as having a single homogeneous form of rDNA as detected by a 8.4 kb *Pst*-1 fragment hybridizing to pMF2 and pCGR1N.

Morphologically variable type 2 strains (see Chapter 2) were also diverse at DNA levels having different forms of rDNA (Figures 3.2). The *PstI-SphI*-and *SstI* digested total DNA blots, show RFLPs for rDNA within type 2 strains (Figures 3.1, 3.2, 3.6, and 3.7). Only the *Hind*III-digested blot shows a similar pattern of rDNA bands for type 2 strains (Figure 3.5). These results suggest that rDNA among type two strains is heterogeneous. In addition, the specific

detection of only type 1 rDNA by pCGR1N suggest that

although some type 2 strains contain a rDNA form similar in

size to type 1 rDNA, the sequences may be different at least

at the NTS region. Several subclones from the NTS region of

type 2 rDNA hybridized to both type 1 and type 2 rDNA.

Therefore, a type 2 strain specific rDNA marker was not

found. Restriction site polymorphisms and length

heterogeneity in the rDNA unit repeat have been commonly

observed in many fungi. These polymorphisms were found

within the NTS region (Verbeet et al. 1983; Cassidy et al.

1984; Russell et al. 1984; Van Heerikhuizen et al 1985;

Chambers et al. 1986; Lachance 1989; Rogers et al. 1989),

ITS region (Chambers et al. 1986; Buckner et al. 1988) or

within coding regions (Chambers et al. 1986; Martin 1990).

In the fruit fly *Drosophila melanogastor* the presence of

introns has given rise to polymorphic forms of rDNA (Glover

and Hogness 1977). In fungi evidence for the presence of

introns in the rDNA coding regions is inconclusive (Buckner

et al. 1988).

Type 1 and type 2 *C. gloeosporioides* strains,

distinguished by rDNA polymorphisms were different both in

cutinase isozymes and molecular organization of relevant DNA

sequences. The slightly acidic pH optimum (pH 6.0) of

cutinolytic activity (Table 3.2) of these strains is a

reflection of their pathogenicity to aerial plant parts

(flowers or leaves) as observed for other aerial plant

pathogens such as *V. inaequalis* (Köller and Parker 1989),
Botrytis cinera (Salinas et al. 1986) and *Cochliobolus
heterostrophus* (Trail and Köller 1990). This pH preference
is also congruent with the hypothesis that pathogens with
this type of cutinase are specialized for infecting aerial
plant surfaces rather than stem bases and roots (Trail and
Köller 1990).

All cutinases are serine esterases, and therefore they
can be detected by ^{3}H-DFP which phosphorylates and inhibits
specific serine esterases (Köller and Kolattukudy 1982;
Kolattukudy 1985; Köller and Parker 1989). The two ^{3}H-DFP
labelled bands (Figure 3.9) which are within the range of
molecular weights of fungal cutinases (Kolattukudy 1980;
Tanabe et al. 1988; Trail and Köller 1990) were not
previously seen in *Colletotrichum* species including papaya
isolate of *C. gloeosporioides* (Dickman et al. 1982;
Kolattukudy 1985). Although molecular weight and culture
conditions prior to electrophoresis suggest that cutinase
enzymes are present, DFP binding is serine esterase but not
cutinase specific (Köller and Kolattukudy 1982; Kolattukudy
1985; Köller 1991). These bands may not represent two
different primary gene products but may be the result of
post-translational modification (Köller 1991; Soliday et al.
1984). Some cutinase enzymes may undergo a proteolytic nick
and appear as two fragments after reduction of a disulfide
bridge and electrophoresis under denaturing conditions

(Köller 1991; Lin and Kolattukudy 1980; Purdy and Kolattukudy 1975b; Soliday and Kolattukudy 1976). Conclusive demonstration that these two bands are actually two cutinases awaits further experiments such as purification and characterization of the catalytic activity. Other fungal pathogens, *N. haematococca* (Purdy and Kolattukudy 1975a, 1975b), *A. brassicola*, and *R.solani* (Trail and Köller 1990) are known to produce at least two distinct cutinases as detected by distinct isozyme bands and enzyme catalysis.

For type 2 strains, only one DNA restriction fragment hybridizes to the cutinase gene probe but two cutinase isozymes may be present. One possibility is that these may be two forms of cutinases encoded by distinctly different genes in the same organism. Only one form of cutinase has been described biochemically from the papaya strain of *C. gloeosporioides* (Dickman et al. 1982). The poor hybridization of type 1 strains to the cutinase clone despite the fact that they have abundant cutinase activity suggests considerable evolutionary diversification of cutinase gene sequences. Since the cDNA clone for cutinase from *C. capsici* hybridizes readily with the total DNA from *C. graminicola* and the *C. gloeosporioides* strain from papaya, these species may be more related to type 2 than type 1.

Another line of evidence for distinct genetic forms of

C. gloeosporioides from citrus comes from PCR amplification of a cutinase gene sequence. The published DNA sequences for cutinase genes from *C. gloeosporioides* and *C. capsici* (Ettinger et al. 1987) show both conserved and diversified regions of the gene. Two regions of identical sequence flank a short stretch of DNA which includes the 52 bp intron of the *C. gloeosporioides* cutinase and the 57 bp intron of the *C. capsici* cutinase. The primer sequences are conserved in cutinase genes of *C. gloeosporioides*, and *C. capsici* and respectively should amplify a 220 bp or 222 bp fragment when used as primers for PCR (Ettinger et al. 1987). Indeed, one or two amplified fragments of about 220-260 bp were obtained from the DNA of the four strains tested. When used as a probe for Southern hybridization these fragments were anticipated to hybridize to conserved elements of the cutinase gene and divergent sequences expected within the intron. However, these sequences hybridize to multiple restriction fragments producing DNA finger prints that correspond to RFLP types.

Hybridization to these probes must not be specific for cutinase sequences. The exact nucleotide sequences of the two amplified fragments were not determined. Therefore, it is necessary to determine the nucleotide sequence of the 220 and 260 bp amplified fragments before reaching any conclusions. However, a computer search of the GenBank database (Release 70.0, December 15, 1991) indicated no

significant sequence similarity between the 220 bp targeted

sequence of the cutinase gene from *C. gloeosporioides*

(Ettinger et al 1987) and genes other than for *C. capsici*

and *C. gloeosporioides* cutinase. Hybridization may be to

sequences common to other introns such as putative splice

junction sequences identified previously (Ettinger et al.

1987). Hybridization to the repetitive sequences may be

detected using the 220 bp probe and not the entire cutinase

clone because the intron sequence represents <1% of the

clone but 25% of the PCR fragment.

Cutinase is not the only extracellular enzyme produced

by fungi in the process of plant infection. There are many

others such as cellulolytic and pectinolytic enzymes

produced by fungi in culture and in diseased leaves (Oke

1989; Prusky et al. 1989). The type 1 and type 2 strains

also produce different isozymes of pectinesterase that

correlate to type 1 and type 2 (personal communication

Gantotti and Davis, Homestead, FL).

DNA polymorphisms detected by hybridization to 3 of 4

"housekeeping" genes from *N. crassa* also correspond directly

to type 1 and type 2 strains. To detect DNA polymorphisms

correlated to type 1 and type 2 strains by Southern

hybridization using these molecular markers it was not

necessary to search for specific restriction enzymes or to

test multiple loci. The only enzyme used to digest total

DNA, *Hind*III was sufficient to provide RFLPs capable of

separating type 1 from type 2 strains. However, exceptions
to the strict correlation between polymorphism and strain
type was seen for hybridization to pNC2. In this case
variation was seen among type 1 strains. On the whole all
the molecular markers suggest that the two types are
different at the molecular level indicating they are indeed
genetically distinct populations of *C. gloeosporioides*.

CHAPTER 4
VARIABILITY OF MOLECULAR KARYOTYPES AND CHROMOSOMAL DNAS IN *COLLETOTRICHUM GLOEOSPORIOIDES*

Introduction

Pulsed Field Gel Electrophoresis

Macromolecules such as nucleic acids and proteins can be separated on the basis of size, charge or conformation by gel-electrophoresis. Schwartz et al. (1982) made use of the relaxation properties (Klotz and Zimm 1972) of large DNA molecules for their separation in agarose gels by using two alternating electric fields known as pulsed field gel electrophoresis (PFGE). A major advance of the pulsed field gel electrophoresis was achieved by Chu et al. (1986). They applied the principles of electrostatics to calculate the voltages needed to generate homogeneous electric fields using multiple electrodes arranged around a closed contour. In this system, contour clamped homogeneous electric field (CHEF) gel electrophoresis, twenty four electrodes were arranged in a hexagonal contour which offers reorientation angles of 60 or 120.

Molecular Karyotypes of Fungi

Fungal chromosomes are too small to be observed readily by conventional cytological methods using light microscopy. However, electrophoretic karyotyping and molecular analysis of chromosome-size DNA have become the new methods for studying genomic structure of various organisms including filamentous fungi. Many studies have conclusively demonstrated that DNAs resolved by PFGE corresponds to chromosomes (Carle and Olson 1985; Orbach et al. 1988; Brody and Carbon 1989; Kayser and Wostemeyer 1991). However, the number of bands need not be equal to the number of chromosomes (Horton and Raper 1991). Electrophoretic analysis of chromosomes provide a very convenient and rapid way of assigning genes to chromosomes and for monitoring entire genomes for any chromosomal rearrangements.

Pulsed field gel electrophoresis has been used to separate chromosome-size DNA and analyze molecular karyotypes of many fungi such as *S. cerevisiae* (Schwartz and Cantor 1984; Chu et al. 1986), *Candida albicans* (Snell and Wilkins 1986), *Schizosaccharomyces pombe* (Smith et al 1987; Vollrath and Davis 1987), *Neurospora crassa* (Orbach et al. 1988), *Ustilago maydis* (Kinscherf and Leong 1988), *Candida stellatoidea* (Kwon-Chung 1988, 1989: Wickes et al. 1991), *Aspergillus nidulans* (Brody and Carbon 1989), *C. gloeosporioides* (Masel et al. 1990), *Ustilago hordei, Tilletia caries, T. controversa* (McCluskey et al. 1990),

Schizophyllum commune (Horton and Raper 1991), *Absidia glauca* (Kayser and Wostemeyer 1991), *Septoria tritici* (McDonald and Martinez 1991), *Nectria haematococca* (Miao et al. 1991), *Acremonium* species (Smith et al. 1991; Walz and Kuck 1991), *Leptosphaeria maculans* (Taylor et al. 1991), and *Fusarium oxysporum* (Momol and Kistler 1992).

Pulsed field gel electrophoresis of chromosomal DNA combined with Southern analysis using linkage group-specific probes were key methods in defining molecular karyotypes of *N. crassa* (Orbach et al. 1988) and *Aspergillus nidulans* (Brody and Carbon 1989). Molecular karyotyping of *N. crassa* by Orbach et al. (1988) confirmed the seven linkage groups previously defined by genetic analysis (Perkins et al. 1982). The genome size of *A. nidulans* was estimated by Brody and Carbon (1989) to be approximately 31 Mb with six chromosome-sized DNA bands. Kayser and Wostemeyer (1991) reported differences in electrophoretic karyotypes for mating types of the Zygomucete *Absidia glauca*.

Molecular karyotypes of many plant pathogenic fungi examined to date have been variable. Kinscherf and Leong (1988) analyzed the molecular karyotype of *U. maydis* and demonstrated that considerable chromosomal length heterogeneity exists in this fungus. DNA hybridization analysis suggested that stable large scale inter-chromosomal exchange has given rise to novel chromosomes in one of the strains. Taylor et al. (1991) using TAFE demonstrated that

the karyotypes of highly virulent and weakly virulant strains of *Leptosphaeria maculans* (black leg of crucifers) were polymorphic in both chromosome number and size. Highly variable karyotypes for *N. haematococca* with unique karyotypes for each strain were reported by Miao et al. (1991). Deletions of large amounts of DNA from chromosomes have given rise to karyotype variation as well as a decreased frequency of the pisatin demethylase gene in *N. haematococca*. Masel et al. (1990) suggested that chromosomal rearrangements may play a role in generating variability of karyotype of *C. gloeosporioides*. Distinct electrophoretic karyotypes were reported for strains from two types of *C. gloeosporioides* causing different anthracnose diseases in *Stylosanthus* species in Australia. The strains showed extensive chromosomal polymorphisms for both length and number in the mini-chromosomes (molecules less than 2 million base pairs (Mb) in length) within each type.

The present study was undertaken to investigate the variation of molecular karyotypes and chromosomal DNAs in two types of *C. gloeosporioides* (see Chapter 2 and 3) causing post bloom fruit drop of Tahiti lime and Sweet orange.

Materials and Methods

Strains of *Colletotrichum gloeosporioides*

Strains used were obtained from several different areas
of Florida, Mexico, and from the Commonwealth Institute of
Mycology, England. They were isolated from diseased lime or
orange tissues. Details of host, place of collection and
date are tabulated in the appendix A.

Preparation of Protoplast Plugs

The strains of *C. gloeosporioides* were grown for 7 days
in 50 ml of 20% (w/v) V-8 juice (Campbell Co., Camden, NJ)
in at 250 rpm in Erlinmayer flasks on a Lab-Line orbit
shaker (Lab-Line Instruments Inc., Melrose Park, IL) at
ambient temperature (21-23 °C), and conidia were collected
by centrifugation at 7000 x g for 5 min. About 10^9 spores
per ml were resuspended in 50 ml potato dextrose broth (PDB)
and incubated at room temperature (23 to 25 °C) for 16-24 h
at 200 rpm. When over 90% of spores were germinated, the
germlings were pelletted by centrifuging at 7000 x g for 5
min. Protoplasts were made by adding germlings to a 10 ml
solution containing NovoZym 234 (Novo Industries, Bagsvaerd,
Denmark) a complex mixture of wall-degrading enzymes. The
NovoZym solution was prepared by mixing 1.5 ml of 1 M
sorbitol, 50 mM sodium citrate containing 0.2 g of NovoZym
234 with 8.5 ml of 1.4 M $MgSo_4$, and 50 mM Sodium Citrate pH

5.8. Germlings were incubated in this solution with gentle rocking on a Bellco rocker (Bellco Biotechnology, Vineland NJ) at 4 rpm for 3 to 6 h at ambient temperature for 3 to 6 hours until most cells were protoplasts. The protoplasts were filtered through 4 layers of cheese cloth in order to remove cell debris and undigested germlings. The filtrate was centrifuged at 3000 rpm for 25 min at room temperature. Protoplasts were removed from the top and washed three times with 1 M sorbitol-50 mM EDTA pH 8.0. Protoplast inserts for PFGE were made as described by method 1 of Orbach et al. (1988).

Electrophoresis and Southern Analysis

A commercially available apparatus (BioRad CHEF DRII, Richmond, CA) using different pulse time combinations was employed in order to separate chromosome-sized DNAs. Electrophoresis was done with 0.6% FastLane agarose (FMC BioProducts, Rockland, ME) gels in 0.25X Tris Borate EDTA (TBE) buffer (Sambrook et al. 1989) at 4 °C with rapid circulation of the buffer. The gels were run at 40 volts for 6-10 days. Pulse times were "ramped" for various times ranging from 10 to 180 min. For the separation of smaller chromosome sized DNA 1% SeaKem Agarose (FMC BioProducts, Rockland, ME) in 0.5X TBE buffer was used. These gels were run at 200 V for 24 h with pulse times of 30-60 s or 50-90s.

Southern hybridization (Appendix C) experiments with ^{32}P labelled ribosomal DNA (pMF2), ß-tubulin gene (pSV50) and cutinase gene (see chapter 3) were carried out to assign these sequences to chromosome-size DNAs separated in CHEF-gels.

Results

Chromosome-size DNAs (henceforth called chromosomes) from type 1 and type 2 strains of *C. gloeosporioides* were separated by PFGE using *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* chromosome size DNA as standards (BioRad Laboratories, Richmond, CA). The sizes in Table 4.1 represent the average size calculated for each chromosome size-DNA band from independent CHEF-gels. Calculated sizes for individual chromosome size DNA bands and relevant figures are compiled in the Appendix D. Type 1 strains have chromosomes distinguishable from type 2 strains (Table 4.1). The chromosomes of *C. gloeosporioides* isolated from *Stylosanthes* have been classified by Masel et al. (1990) into larger, similar-sized chromosomes (>2 Mb) and smaller variable-sized elements called "minichromosomes" (<2 Mb). A similar arrangement was noted for strains isolated from Tahiti lime and Sweet orange. Type 1 strains possess 5 chromosomes (Figures 4.1 and 4.2) and an additional 1 or 2 minichromosomes (Figure 4.3 and 4.5). Type 2 strains possess 3 chromosomes (Figures 4.1 and 4.2) in addition to 2 to 4

Table 4.1 Estimated megabase sizes for chromosome-size DNA
from *Colletotrichum gloeosporioides* type 1 and
type 2 strains

Strain	Estimated size[*] (Mb)						
	I	II	III	IV	V	VI	VII
Type-1 strains							
H-1	7.6	7.0	4.7	3.7	3.3	1.1	0.63
H-3	7.6	7.0	4.7	3.7	3.3	1.1	0.63
H-9	7.6	7.0	4.7	3.7	3.3	1.1	0.63
H-25B	7.6	7.0	4.7	3.7	3.3	1.1	0.63
H-36	7.6	7.0	4.7	3.7	3.3	0.77	0.63
LP-1	7.6	7.0	4.7	3.7	3.3	1.6	0.63
Maran	7.6	7.0	4.7	3.7	2.8	-	0.63
IMB-3	7.6	7.0	4.7	3.7	3.3	1.1	0.63
OCO	7.6	7.0	4.7	3.7	2.8	-	0.65
Type-2 strains							
H-4	7.8	4.7	3.7	0.42	0.38	-	-
H-12	7.8	4.7	3.7	0.46	0.38	-	-
H-46	7.8	4.7	3.7	0.52	0.47	0.42	0.27
H-48	7.8	4.7	3.7	0.46	0.43	0.40	-
180269	7.8	4.7	3.7	0.43	0.41	0.39	-
226802	7.8	4.7	3.7	0.44	0.42	0.39	0.37

[*]*Schizosaccharomyces pombe* and *Saccharomyces cerevisiae* size
standard were used for the calculation of Mb sizes. Megabase
sizes greater than 5.6 were estimated by extending the
calibration curve and therefore may be considered
approximate sizes.
- = not detected in any of the gels.

minichromosomes (Figures 4.4 and 4.5) depending on the strain. Within each type, strains show variations in chromosome number and size. However type 2 strains show more total variation in chromosome and minichromosome size (Figures 4.4 and 4.5).

A Southern blot separating larger chromosome-size DNAs was hybridized with a ^{32}P labelled ribosomal DNA probe. The rDNA is associated with the 4.7 Mb chromosome in type 1 strains and with the 7.8 Mb chromosome in type 2 strains (Figure 4.6). The cutinase gene can be assigned to the 4.7 Mb chromosome-size DNA only in type-2 strains (Figure 4.7). Homologous regions were not detected in chromosomes of type 1 strains. The ß-tubulin gene hybridizes to both the 7.0 and 7.6 Mb chromosome doublet in type-1 strains and the 7.8 Mb chromosome in type-2 strains (results are not shown due to weak signals on X-ray film).

Figure 4.1 Chromosome-sized DNAs from *C. gloeosporioides*
compared to those for yeast (*S. cerevisiae*) and
fission yeast (*S. pombe*). DNAs were separated on
agarose gels using CHEF electrophoresis. Gels
were stained with ethidium bromide (0.5 μg/ml)
and fluorescence photographed with UV
transillumination. DNAs from the indicated
strains were run in 0.25X TBE, 0.6% agarose for
168 h at 40 V. Pulse switching times were ramped
from 40-70 min. Numbers at the left indicate size
standards in megabases (Mb).
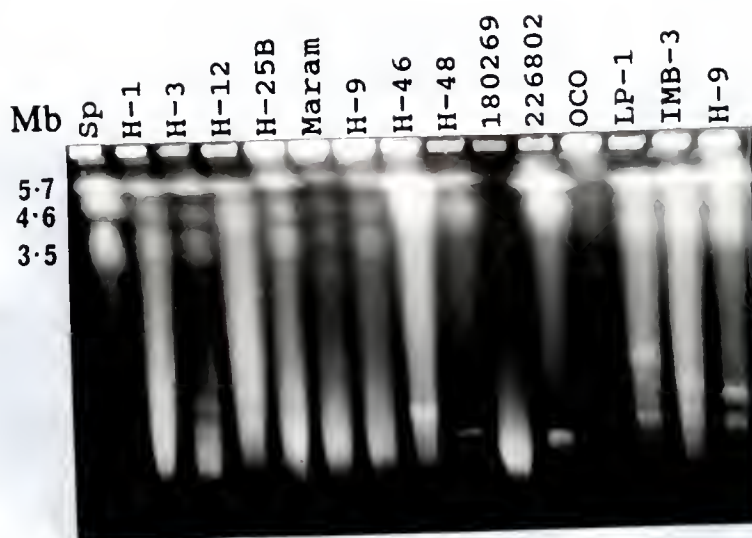
Figure 4.2 Chromosome-sized DNAs from *C. gloeosporioides* compared to those for fission yeast (*S. pombe*). DNAs were separated on agarose gels using CHEF electrophoresis. Gels were stained with ethidium bromide (0.5 µg/ml) and fluorescence photographed with UV transillumination. DNAs from the indicated strains were run in 0.25X TBE, 0.6% agarose for 168 h at 40 V. Pulse switching times were ramped from 20-60 min. Numbers at the left indicate size standards in megabases (Mb).
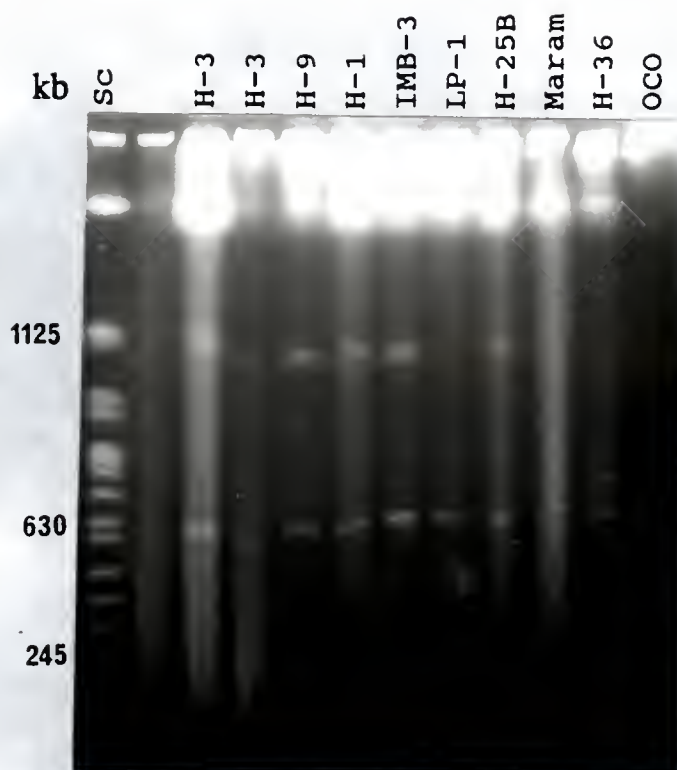
Figure 4.3 Chromosome-sized DNAs from *C. gloeosporioides* compared to those for yeast (*S. cerevisiae*). DNAs were separated on agarose gels using CHEF electrophoresis. Gels were stained with ethidium bromide (0.5 μg/ml) and fluorescence photographed with UV transillumination. DNAs from the indicated strains were run in 0.5X TBE, 1.0% agarose for 24 h at 200 V. Pulse switching times were ramped from 60-90 s. Numbers at the left indicate size standards in kilobases (kb).
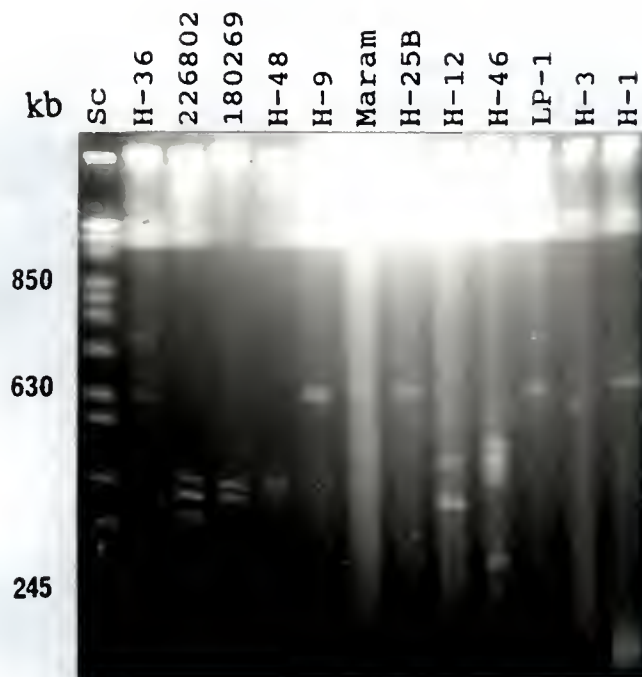
Figure 4.4 Chromosome-sized DNAs from *C. gloeosporioides*
compared to those for yeast (*S. cerevisiae*). DNAs
were separated on agarose gels using CHEF
electrophoresis. Gels were stained with ethidium
bromide (0.5 µg/ml) and fluorescence photographed
with UV transillumination. DNAs from the
indicated strains were run in 0.5X TBE, 1.0%
agarose for 24 h at 200 V. Pulse switching times
were ramped from 30-60 s. Numbers at the left
indicate size standards in kilobases (kb).
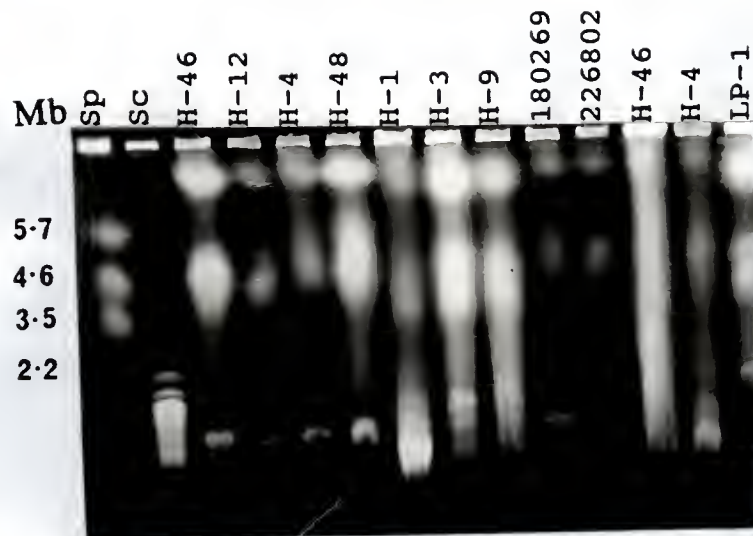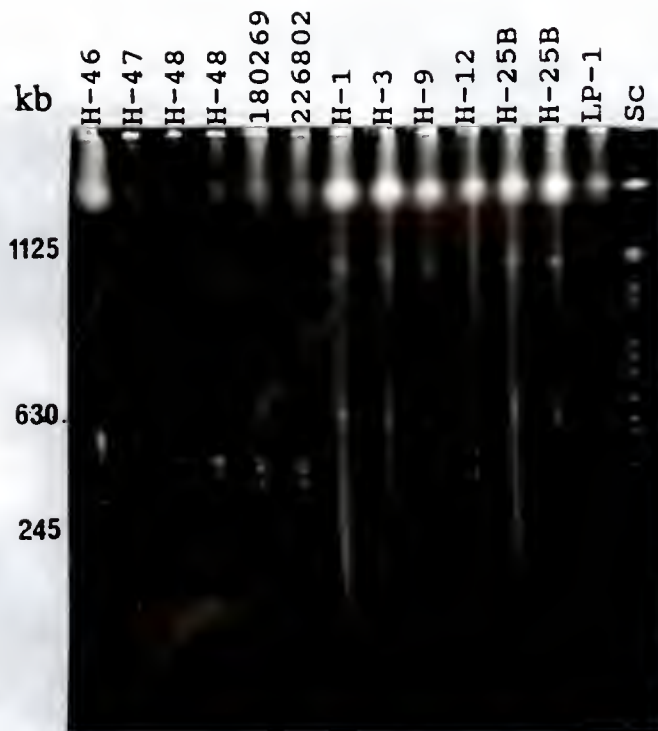
Figure 4.5 Chromosome-sized DNAs from *C. gloeosporioides* compared to those for yeast (*S. cerevisiae*). DNAs were separated on agarose gels using CHEF electrophoresis. Gels were stained with ethidium bromide (0.5 μg/ml) and fluorescence photographed with UV transillumination. DNAs from the indicated strains were run in 0.5X TBE, 1.0% agarose for 24 h at 200 V. Pulse switching times were 60 s (15 h) and 90 s (9 h). Numbers at the left indicate size standards in kilobases (kb).
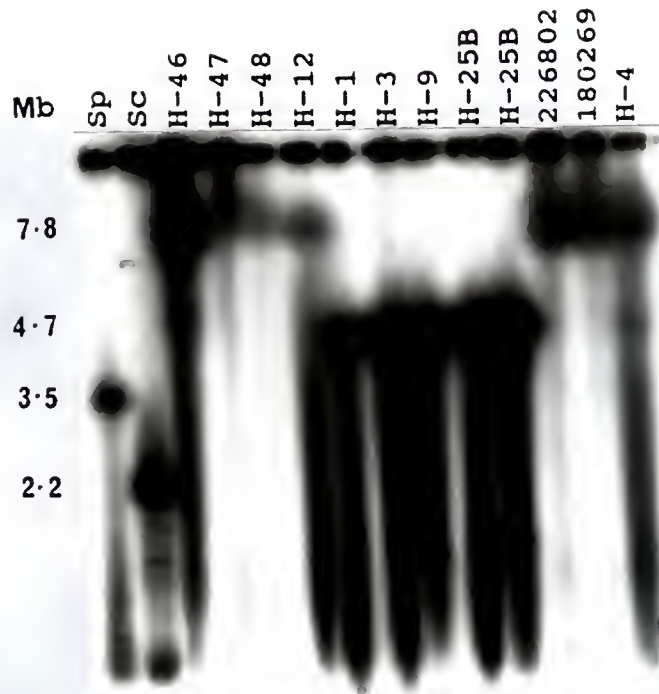
Figure 4.6 Southern blot of chromosome-sized DNAs from *C. gloeosporioides* strains, yeast (*S. cerevisiae*) and fission yeast (*S. pombe*) hybridized to a ^{32}P labelled clone containing the ribosomal DNA repeat from *N. crassa*. Hybridization is to a 4.7 or 7.8 Mb for type 1 or type 2 strains respectively. Gel running conditions were 0.25X TBE, 0.6% agarose for 264 h at 40 V. Pulse switching times were ramped from 10-120 min. Numbers at the left indicate size in megabases (Mb).
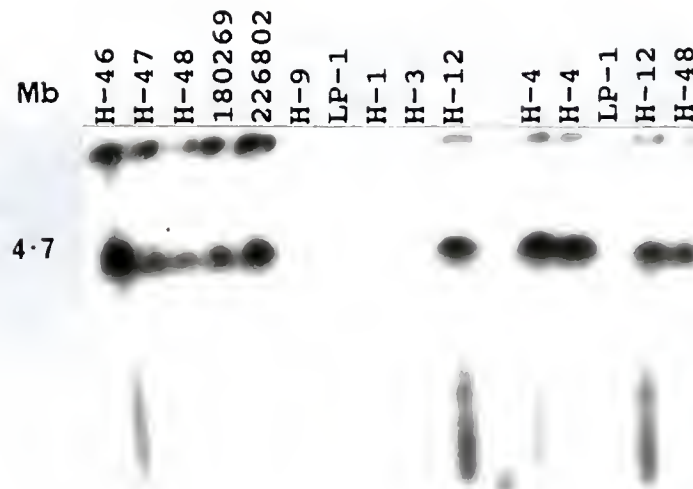
Figure 4.7 Southern blot of chromosome-sized DNAs from *C. gloeosporioides* strains, hybridized to a ^{32}P labelled clone containing the cutinase gene. Hybridization is to a 4.7 Mb for type 2 strains. Gel running conditions were 0.25X TBE, 0.6% agarose for 240 h at 40 V. Pulse switching times were 40 min (120 h) and 100 min (120 h).

Discussion

In previous studies, the DNAs resolved in agarose gels
by PFGE corresponded to chromosomes (Carle and Olson 1985;
Orbach et al 1988; Brody and Carbon 1989; Kayser and
Waltsmeyer 1991). The number of bands visible in an ethidium
bromide stained gel may not always be equal to the number of
chromosomes. Sometimes different chromosomes of same size
may co-migrate resulting in a single band (Brody and Carbon
1989; Horton and Raper 1991). In general the variation in
the number and migration lengths of these bands may
represent differences in size and number of chromosomes in
the organism. Chromosomal variations produced by
translocations, recombinations and losses in fungi may be
easily studied by electrophoretic karyotyping.

Pulsed field gel electrophoresis has allowed detection
of chromosomal variation within species of phytopathogenic
fungi (Kinscherf and Leong 1988; Kistler and Miao 1992;
Masel et al. 1990). Similarly, variation in chromosome-sized
DNA in *C. gloeosporioides* from Tahiti lime and Sweet orange
has been observed. Two distinct electrophoretic patterns for
type 1 and type 2 can be described (Table 4.1). These
patterns show similarities to those described by Masel et al
(1990) for type-A and type-B strains of *C. gloeosporioides*
from *Stylosanthus*. Similar to type-A, the type 1 strains
which have 5 large chromosomes, whereas type-B is similar to
type 2 strains by having 3 large chromosomes.

Minichromosomes are variable and within each type there are differences in number and size. Type 2 strains have a greater diversity in size and number of minichromosomes. The differences in the size of the larger chromosomes between types is best illustrated by the differently sized molecules hybridizing to pMF2 and cutinase gene. The smaller chromosomal DNA observed in these strains may be similar to B chromosomes (Jones and Rees 1982). In other fungi, B chromosomes have been shown to contain unique pathogenicity or virulence genes (Miao et al. 1991) or simply duplicated sequences from larger chromosomes (Rikkerink et al. 1990).

CHAPTER 5
GENERAL DISCUSSION AND CONCLUSIONS

The objectives of these studies were to examine variability of *Colletotrichum gloeosporioides* from Tahiti lime and Sweet orange at the morphological, molecular, and chromosomal levels.

Morphological variability of *C. gloeosporioides* is reported in Chapter 2. These results are comparable to morphological observations made by Burger (1921), Fagan (1980), Denham and Waller (1981), and Sonoda and Pelosi (1988). A reasonable level of morphological and growth differences exist between type 1 and type 2 strains. By observing mycelial color, growth rate, and sectoring in potato dextrose agar and potato dextrose broth, one may be able to easily recognize type-1 and type-2 strains at morphological level.

The two sub-populations recognized in Chapter 2 show their phenotypic and genetic distinctiveness as reported in Chapter 2 and 3. The differential sensitivity to benomyl of type 1 and type 2 strains has an important practical aspect. The present use of benomyl as a fungicide to control PFD needs to be reconsidered in light of benomyl tolerance of virulent type-1 strains. All the strains were equally able

to infect persian lime flowers in this study. Type 1 may be considered as the most virulent form based on observations made by others (Fagan 1980; Sonoda and Pelosi 1988; Agostini et al 1992; Gantotti and Davis, personal communication).

The isozyme and genetic diversity (Chapter 3) of cutinases between type 1 and type 2 strains needs to be further investigated. Sequencing of cutinase proteins and genes may provide accurate measurement of diversity of these phenotypic and genetic characters between type 1 and type 2 strains.

The ribosomal DNA in *C. gloeosporioides* is polymorphic (Chapter 3). Within type 1 strains, rDNA appears to be of a single form. The sequence of type 1 rDNA differs from type 2. The type 1 rDNA specific subclone pCGR1N may be a suitable probe to detect these strains from PFD epidemics. The cloning and characterization of rDNA from type 2 strains was incomplete. Only a 6.8 kb *Pst*I fragment was cloned and mapped as a form of type 2 rDNA. Observations made in this study suggest that rDNA may exist in several forms in morphologically unstable type 2 strains. Other forms of type 2 rDNA need to be cloned and mapped. Perhaps, a sequence comparison at the NTS region may provide some insight into the rDNA diversity within type 2 strains and between type 1 and type 2 strains. In addition, the RFLP patterns associated with four "house keeping genes" also suggest genetic diversity within type 1 or type 2 strains.

The specific RFLP detected by the ß-tubulin gene needs to be further investigated in light of the differences in benomyl tolerance of type 1 and type 2 strains (Chapters 2 and 3). Future research may need to be focused on cloning the ß-tubulin genes from type 1 and type 2 strains. A sequence comparison may perhaps elucidate the molecular mechanisms underlying the observed differences in benomyl tolerance.

The distinct molecular karyotypes (Chapter 3) representing each subpopulation of *C. gloeosporioides* confirm the division of two RFLP types. The two types of strains differ not only in chromosome number and size but also in chromosome assignment.

On the whole, all morphological, phenotypic and genetic markers suggest that type 1 strains are distinct from type 2. The taxonomic position of these type 1 and type 2 strains is not known. They may be considered as subspecies or races within the species. However, host specificity corresponding to types was not observed.

This study has conclusively demonstrated that two genetically distinct pathogen populations of *C. gloeosporioides* exist in association with post bloom fruit drop disease of Tahiti lime and Sweet orange. Strains from both types are present in Florida. However, of the five strains obtained from elsewhere outside the United States all were type 2.

APPENDIX A
STRAINS OF *COLLETOTRICHUM GLOEOSPORIOIDES*

Strain	Year & place	Host
Type 1		
H-1	1989, Immokalee, FL	Tahiti lime
H-3	1989, Immokalee, FL	Tahiti lime
H-9	1988, Homestead, FL	Tahiti lime
H-21	1989, Homestead, FL	Tahiti lime
H-22	1989, Homestead, FL	Tahiti lime
H-25B	1989, Homestead, FL	Tahiti lime
H-35[a]	1989, Ocala, FL	Sweet orange
H-36[a]	1989, Ft. pierce, FL	Sweet orange
LP-1[c]	1990, Lake Placid, FL	sweet orange
Maran[c]	1990, Indiantown, FL	Sweet orange
OCO[c]	1990, Arcadia, FL	Sweet orange
TUR-1[c]	1990, Lake Alfred, FL	Sweet orange
Type 2		
H-4	1989, Immokalee, FL	Tahiti lime
H-11	1988, Homestead, FL	Tahiti lime
H-12	1988, Homestead, FL	Tahiti lime

Strain	Year & place	Host
H-23	1989, Homestead, FL	Tahiti lime
H-24	1989, Homestead, FL	Tahiti lime
H-46[a]	1989, Vera Cruz, Mexico	Sweet orange
H-47[a]	1989, Vera Cruz, Mexico	Sweet orange
H-48[a]	1989, Vera Cruz, Mexico	Sweet orange
180269[b]	Belize	Sweet orange
226802[b]	Belize	Sweet orange

[a]Strains were provided by Dr. R. Sonoda, University of Florida, IFAS Agricultural Research and Education Center, Fort Pierce, FL.
[b]Strains obtained from Commonwealth Institute of Mycology, Kew, London, UK; provided by Dr. R. Sonoda
[c]Strains provided by Dr. L. W. Timmer, University of Florida, IFAS Citrus Research and Education Center, Lake Alfred, FL. All the other strains were obtained from the culture collection at University of Florida, IFAS Tropical Research and Education Center, Homestead, FL.

APPENDIX B
ANALYSIS OF VARIANCE TABLES

Table B.1 ANOVA for growth rates (cm/24h) of type 1 and type 2 strains in potato dextrose agar (experiment 1)

Source	df	SS	MS	F_o
Type-1 vs Type-2	1	0.45	0.45	2250[***]
Within Type-1	7[a]	0.2759	0.0394	197[***]
Within type-2	7	0.0225	0.0032	16[***]
Error	64	0.0172	0.0002	
Total	79	0.7656		

[a]9 type 1 isolates were tested. Two isolates OCO and H-36 had identical measurements, and were considered as one for analysis.
*** Significant at $\alpha=0.01$ level

Table B.2 ANOVA for growth rates (cm/24h) of type 1 strains
in benomyl treated potato dextrose agar
(experiment 1)

Source	df	SS	MS	F_0
Strains	7^a	0.2193	0.0313	3130[***]
Concentration	2	0.2401	0.12	12000[***]
Strain X Conc.	14	0.1199	0.0085	850[***]
Error	96	0.0014	0.00001	
Total	119	0.5807		

[a]9 type 1 isolates were tested. Two isolates OCO and H-36
had identical measurements, and were considered as one for
analysis.
*** Significant at $\alpha=0.01$ level

Table B.3 ANOVA for growth rates (mm/h) of type 1 and type
2 strains in potato dextrose agar (experiment 2)

Source	df	SS	MS	F_o
Type-1 vs Type-2	1	0.0367	0.0367	29424[***]
Within Type-1	7[a]	0.0194	0.00277	2216[***]
Within type-2	7	0.0034	0.000485	388[***]
Error	16	0.00002	0.00000125	
Total	31	0.0596		

[a] 9 type 1 isolates were tested. Two isolates OCO and H-36
had identical measurements, and were considered as one for
analysis.
*** Significant at $\alpha=0.01$ level

Table B.4 ANOVA for growth rates (mm/h) of type 1 strains in benomyl treated potato dextrose agar (experiment 2)

Source	df	SS	MS	F_o
Strains	7[a]	0.015	0.002	10***
Concentration	2	0.017	0.008	40***
Strain X Conc.	14	0.004	0.0003	1.5*
Error	24	0.005	0.0002	
Total	47	0.041		

[a] 9 type 1 isolates were tested. Two isolates OCO and H-36 had identical measurements, and were considered as one for analysis.
*** Significant at $\alpha=0.01$ level
* Significant at $\alpha=0.25$ level

Table B.5 ANOVA for growth rates (mm/h) of type 1 and type 2 strains in potato dextrose agar (experiment 1&2 combined)

Source	df	SS	MS	F_o
Type-1 vs Type-2	1	0.10492	0.10492	2098[***]
Within Type-1	7[a]	0.07086	0.01012	202[***]
Within type-2	7	0.00235	0.00033	6.6[***]
Error	96	0.00487	0.00005	
Total	111	0.18301		

[a] 9 type 1 isolates were tested. Two isolates OCO and H-36 had identical measurements, and were considered as one for analysis.
*** Significant at $\alpha=0.01$ level

Table B.6 ANOVA for growth rates (mm/h) of type 1 strains
in benomyl treated potato dextrose agar
(experiment 1 & 2 combined)

Source	df	SS	MS	F_0
Strains	7[a]	0.05440	0.00777	4450***
Concentration	2	0.06265	0.03132	17938***
Strain X Conc.	14	0.03175	0.00226	1294***
Error	145	0.000253	0.00000174	
Total	167	0.014906		

[a]9 type 1 isolates were tested. Two isolates OCO and H-36
had identical measurements, and were considered as one for
analysis.
*** Significant at $\alpha=0.01$ level

APPENDIX C
PROCEDURES FOR DNA LABELLING AND SOUTHERN HYBRIDIZATION

Probes were labelled with either radioactive α-^{32}P or digeoxigenin-labelled deoxyuridine-triphosphate (dUTP) (Boehringer Mannheim Corp. Indianapolis, Indiana) by random priming according to the manufacturer's directions.

Gels were irradiated with UV light (254 nm) for 1-2 min before incubating with 0.25 M HCl for 20 min. DNA was denatured in 0.5 M NaOH-1.5 M NaCl and then neutralized in 0.5 M Tris-HCl-1.5 M NaCl pH 7.0 for 30 m each. DNA was capillarily transferred (Southern 1975) to a Nytran membrane (Schleicher & Schuell, Keene, NH) for >24 h in 10X SSC (1 x SSC=0.5 M NaCl-0.015 M Sodium Citrate pH 7.0) transfer buffer. DNA on the membrane was immobilized by UV cross linking (Stratalinker, Stratagene Co., La Jolla, CA).

Labelling of the probes with ^{32}P dCTP was according to the manufacturer's recommendations (Boehringer Mannheim Corp. Indianapolis, IN). Labelled nucleotide was incorporated into probes by random oligonucleotide priming of the large subunit of DNA polymerase (Klenow fragment). Non-radioactive DNA labelling and detection were by enzyme-linked immunoassay of digeoxigenin-labelled deoxyuridine triphosphate incorporated into probes by random

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oligonucleotide priming of the Klenow fragment.

Membranes were pre-hybridized and hybridized according to the manufacturer's instructions for the nonradioactive process (Boehringer Mannheim Corp. Indianapolis, IN).

For the radioactive high stringency process, membranes were pre-hybridized in sealed plastic bags for 3-6 h at 68 C in 0.2 ml/cm^2 of pre-hybridization solution (250 mg dried milk, 10 μg denatured salmon sperm DNA in 100 ml 6X SSC). After removing pre-hybridization solution 0.1 ml/cm^2, hybridization solution (pre-hybridization solution + 10% w/v dextran sulfate) containing labelled probe was added, and hybridized at 68 °C for $\geq$18 h in sealed plastic bags.

Hybridized blots were washed twice (first, 0.25 g dried milk, 0.1% SDS in 100 ml 2X SSC; second 0.1X SSC, 0.1% SDS) at 68 °C for 45 min each. The blots were air dried and exposed to X-ray films (Kodak X-Omat GR, Kodak co., Rochester NY) at -80 °C.

The following are the low stringency conditions for both radioactive and non radioactive probes: Pre-hybridization was the same. Hybridization was at 65 °C and washes were at 55 °C. Solution for the first wash was same as high stringency conditions. Only the second wash was at 2x SSC instead of 0.1x SSC.

APPENDIX D
CALCULATED MB SIZES FOR CHROMOSOMAL DNAS IN
COLLETOTRICHUM GLOEOSPORIOIDES

In all figures Sp=*Schizosaccharomyces pombe*;
Sc=*Saccharomyces cerevisiae*. All CHEF-gels were run in a
cold room at 4 °C. Gels in figures D.1 to D.7 were 0.6%
FastLane agarose (FMC BioProducts, Rockland, ME). The
running buffer was 0.25X TBE, voltage was 40 V, and the
running time was 7-10 d. Gels in figures D.10 and D.11 were
of 1% SeaKem agarose (FMC BioProducts, Rockland, ME) in 0.5X
TBE buffer at 200 V. Running time was 24 h. The beginning
and ending pulse times are indicated under the figure
legends.

The megabase sizes tabulated in this appendix were
calculated from calibration curves developed for each gel
seperately using *S. pombe* and *S. cerevisiae* chromosomes as
size standards.

Figure D.1 Chromosome-sized DNAs from *C. gloeosporioides* compared to those for yeast (*S. cerevisiae*) and fission yeast (*S. pombe*). DNAs were separated on agarose gels using CHEF electrophoresis. Gels were stained with ethidium bromide (0.5 μg/ml) and fluorescence photographed with UV transillumination. DNAs from the indicated strains were run in 0.25X TBE, 0.6% agarose for 168 h at 40 V. Pulse switching times were ramped from 50-100 min. Numbers at the left indicate size standards in megabases (Mb). Lanes Sp., H-12, Maram and H-36 DNA was partially degraded.

Figure D.2 Chromosome-sized DNAs from *C. gloeosporioides*
compared to those for yeast (*S. cerevisiae*) and
fission yeast (*S. pombe*). DNAs were separated on
agarose gels using CHEF electrophoresis. Gels
were stained with ethidium bromide (0.5 μg/ml)
and fluorescence photographed with UV
transillumination. DNAs from the indicated
strains were run in 0.25X TBE, 0.6% agarose for
240 h at 40 V. Pulse switching times were ramped
from 10-180 min. Numbers at the left indicate
size standards in megabases (Mb).

Figure D.3 Chromosome-sized DNAs from *C. gloeosporioides* compared to those for yeast (*S. cerevisiae*) and fission yeast (*S. pombe*). DNAs were separated on agarose gels using CHEF electrophoresis. Gels were stained with ethidium bromide (0.5 µg/ml) and fluorescence photographed with UV transillumination. DNAs from the indicated strains were run in 0.25X TBE, 0.6% agarose for 240 h at 40 V. Pulse switching times were 40 min (120 h) and 100 min (120 h). Numbers at the left indicate size in megabases (Mb).

Figure D.4 Chromosome-sized DNAs from *C. gloeosporioides* compared to those for yeast (*S. cerevisiae*) and fission yeast (*S. pombe*). DNAs were separated on agarose gels using CHEF electrophoresis. Gels were stained with ethidium bromide (0.5 μg/ml) and fluorescence photographed with UV transillumination. DNAs from the indicated strains were run in 0.25X TBE, 0.6% agarose for 240 h at 40 V. Pulse switching times were ramped from 30-70 min. Numbers at the left indicate size standards in megabases (Mb).

Figure D.5 Chromosome-sized DNAs from *C. gloeosporioides* compared to those for yeast (*S. cerevisiae*) and fission yeast (*S. pombe*). DNAs were separated on agarose gels using CHEF electrophoresis. Gels were stained with ethidium bromide (0.5 μg/ml) and fluorescence photographed with UV transillumination. DNAs from the indicated strains were run in 0.25X TBE, 0.6% agarose for 240 h at 40 V. Pulse switching times were ramped from 30-50 min. Numbers at the left indicate size standards in megabases (Mb).
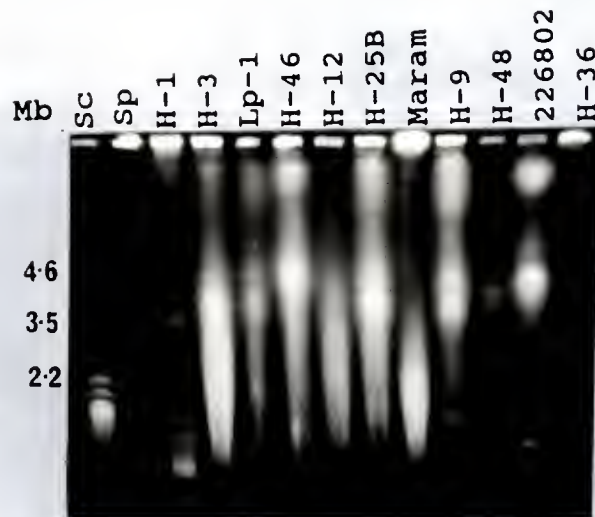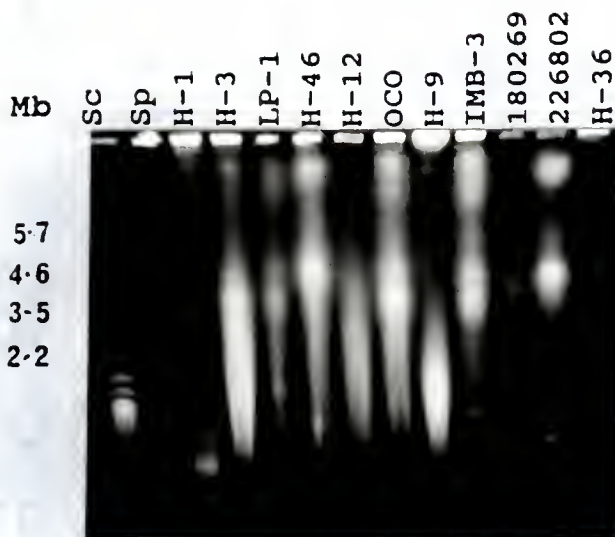
Figure D.6 Chromosome-sized DNAs from *C. gloeosporioides*
compared to those for yeast (*S. cerevisiae*) and
fission yeast (*S. pombe*). DNAs were separated on
agarose gels using CHEF electrophoresis. Gels
were stained with ethidium bromide (0.5 μg/ml)
and fluorescence photographed with UV
transillumination. DNAs from the indicated
strains were run in 0.25X TBE, 0.6% agarose for
168 h at 40 V. Pulse switching times were ramped
from 50-100 min. Numbers at the left indicate
size standards in megabases (Mb). Lanes H-1, H-
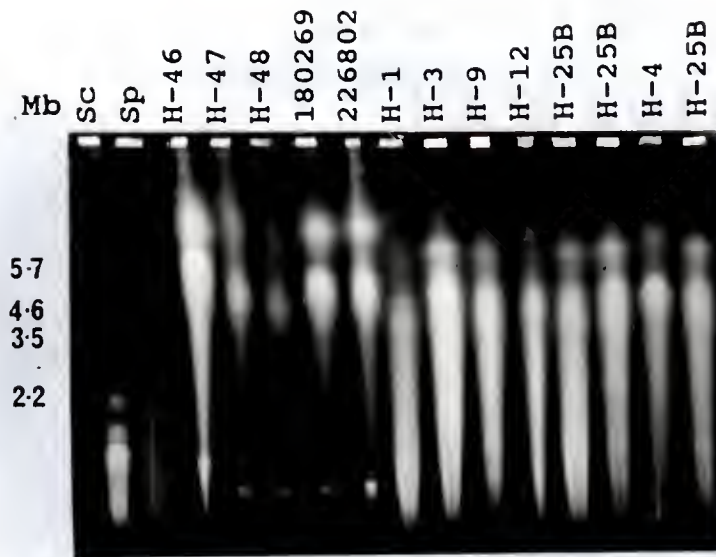H-12, H-9, and 226802 DNA was partially degraded.
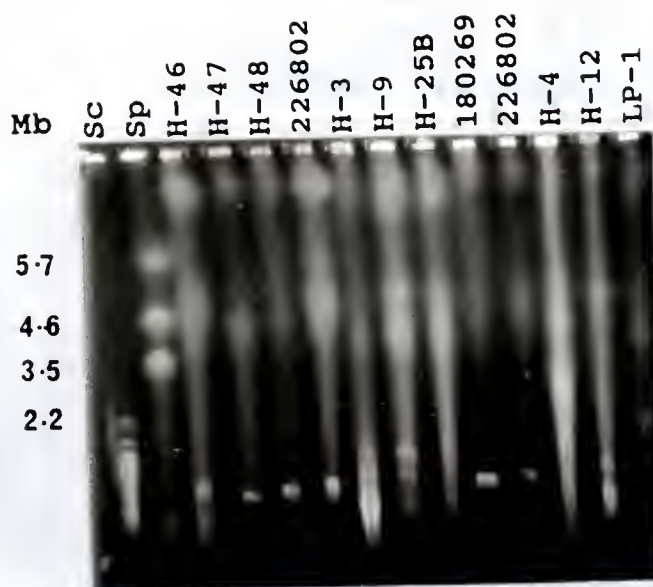
Figure D.7 Chromosome-sized DNAs from *C. gloeosporioides*
compared to those for yeast (*S. cerevisiae*) and
fission yeast (*S. pombe*). DNAs were separated on
agarose gels using CHEF electrophoresis. Gels
were stained with ethidium bromide (0.5 µg/ml)
and fluorescence photographed with UV
transillumination. DNAs from the indicated
strains were run in 0.25X TBE, 0.6% agarose for
192 h at 40 V. Pulse switching times were ramped
from 40-100 min. Numbers at the left indicate
size standards in megabases (Mb). Lane H-4 DNA
was partially degraded.
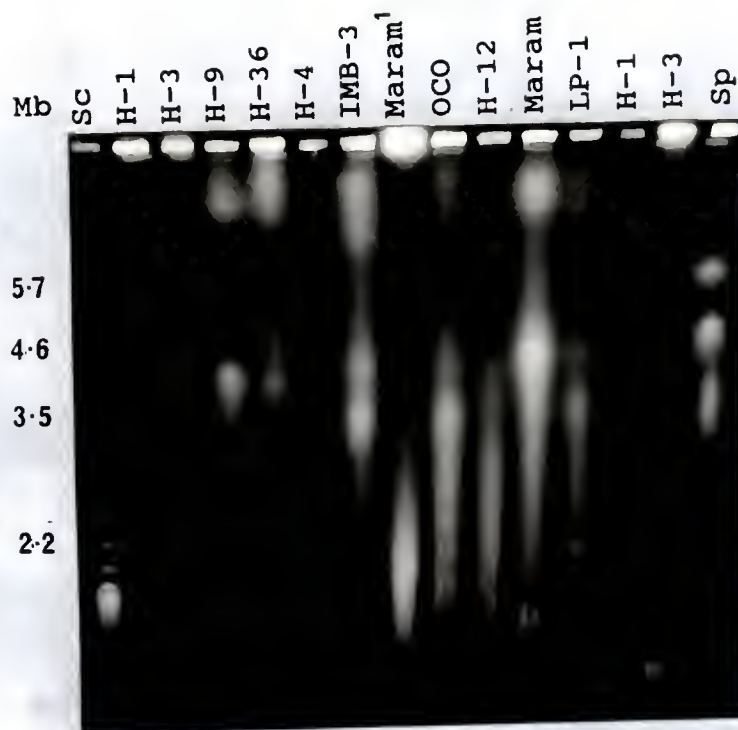
Figure D.8 Chromosome-sized DNAs from *C. gloeosporioides*
compared to those for yeast (*S. cerevisiae*) and
fission yeast (*S. pombe*). DNAs were separated on
agarose gels using CHEF electrophoresis. Gels
were stained with ethidium bromide (0.5 μg/ml)
and fluorescence photographed with UV
transillumination. DNAs from the indicated
strains were run in 0.25X TBE, 0.6% agarose for
192 h at 40 V. Pulse switching times were ramped
from 40-100 min. Numbers at the left indicate
size standards in megabases (Mb). Lanes H-1, H-3,
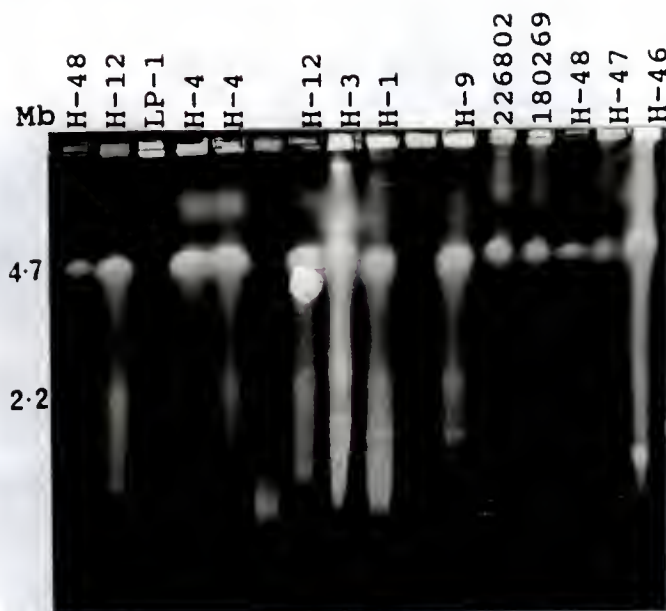H-4, Maram[1], H-12, DNA was partially degraded.
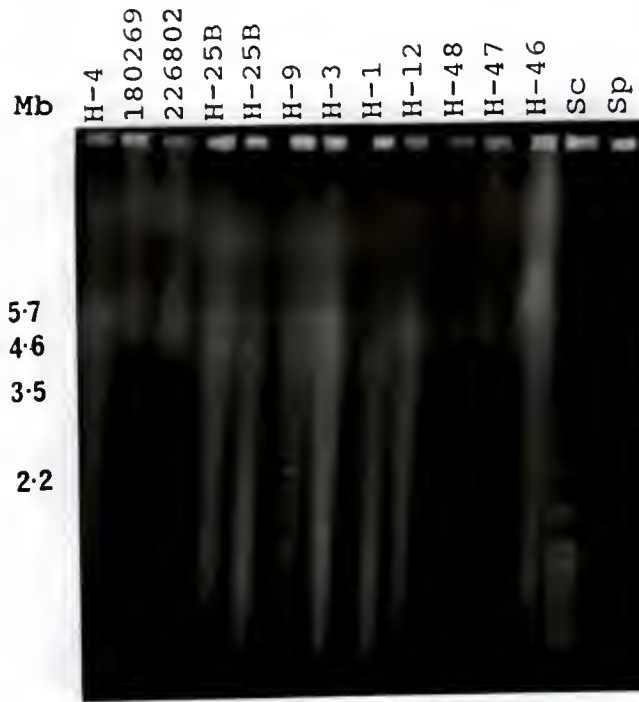
Figure D.9 Chromosome-sized DNAs from *C. gloeosporioides* compared to those for yeast (*S. cerevisiae*) and fission yeast (*S. pombe*). DNAs were separated on agarose gels using CHEF electrophoresis. Gels were stained with ethidium bromide (0.5 μg/ml) and fluorescence photographed with UV transillumination. DNAs from the indicated strains were run in 0.25X TBE, 0.6% agarose for 264 h at 40 V. Pulse switching times were ramped from 10-120 min. Numbers at the left indicate size standards in megabases (Mb). This gel was used in the Figure 4.6.
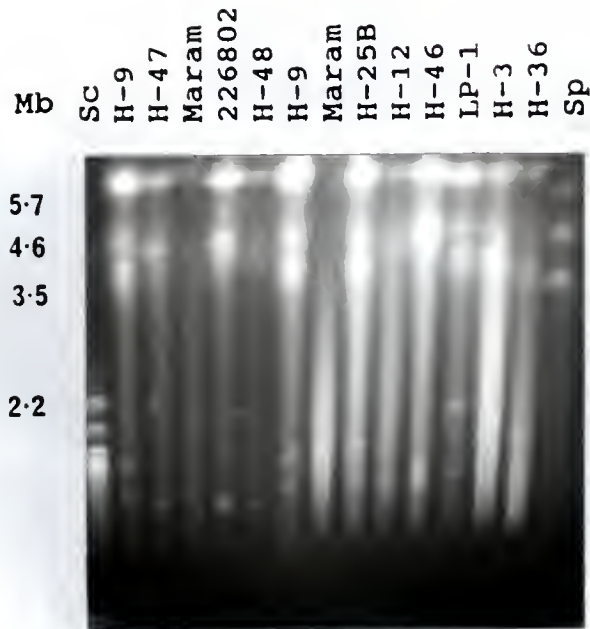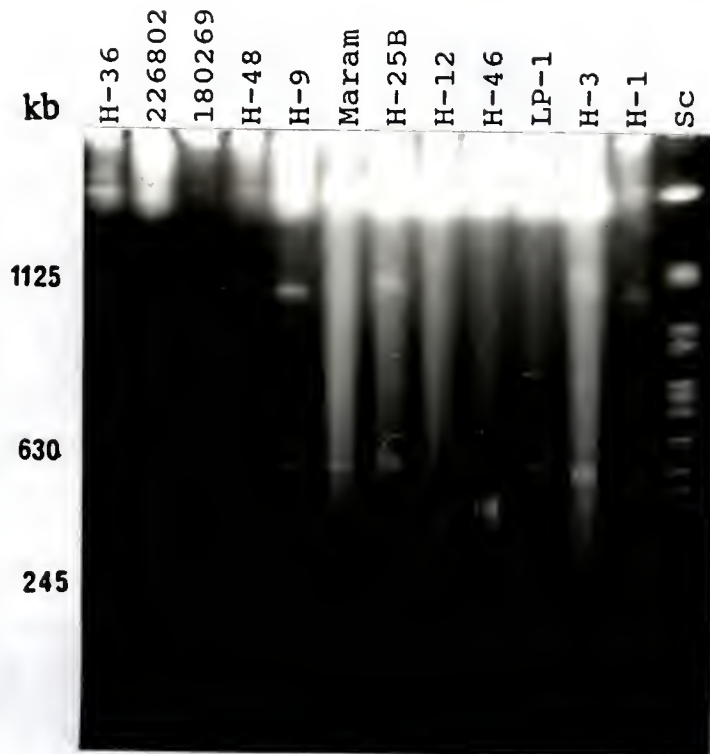
Figure D.10 Chromosome-sized DNAs from *C. gloeosporioides*
compared to those for yeast (*S. cerevisiae*) and
fission yeast (*S. pombe*). DNAs were separated on
agarose gels using CHEF electrophoresis. Gels
were stained with ethidium bromide (0.5 μg/ml)
and fluorescence photographed with UV
transillumination. DNAs from the indicated
strains were run in 0.5X TBE, 1.0% agarose for
24 h at 200 V. Pulse switching times were ramped
from 50-90 s. Numbers at the left indicate size
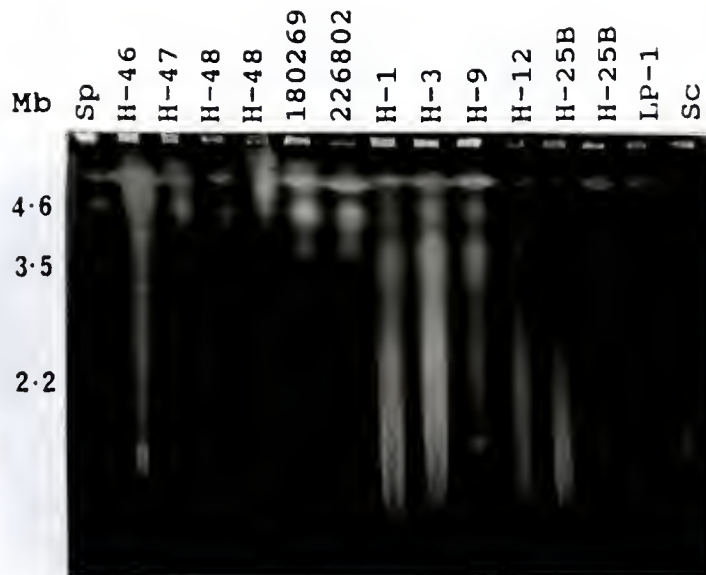standards in kilobases (kb).
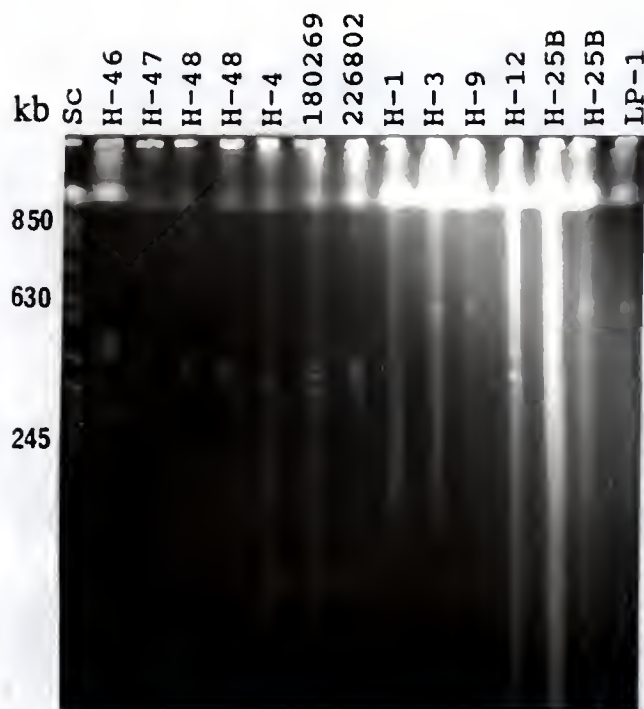
Figure D.11 Chromosome-sized DNAs from *C. gloeosporioides* compared to those for yeast (*S. cerevisiae*) and fission yeast (*S. pombe*). DNAs were separated on agarose gels using CHEF electrophoresis. Gels were stained with ethidium bromide (0.5 μg/ml) and fluorescence photographed with UV transillumination. DNAs from the indicated strains were run in 0.5X TBE, 1.0% agarose for 18 h at 200 V. Pulse switching times were ramped from 30-60 s. Numbers at the left indicate size standards in kilobases (kb).

Table D.1 Estimated magabase sizes for chromosome size DNA

Strain	I	II	III	Chromosome number IV	V	VI	VII	VII	Figure
H-1									
	7.6	7.0	4.9	–	–	–	–	–	4.1
	>5.7	–	4.5	3.7	3.3	–	–	–	4.2
	–	–	–	–	–	1.1	0.63	–	4.3
	–	–	–	–	–	–	0.63	–	4.4
	–	–	–	–	–	1.1	0.63	–	4.5
	7.6	–	4.8	–	–	–	–	–	D.2
	7.6	7.1	4.7	–	–	–	–	–	D.3
	>5.7	–	4.6	3.7	3.4	–	–	–	D.5
	–	–	–	–	–	1.1	0.61	–	D.10
	–	–	–	–	–	–	0.61	–	D.11
H-3									
	7.6	7.0	4.9	–	–	–	–	–	4.1
	>5.7	–	4.5	3.7	3.3	–	–	–	4.2
	–	–	–	–	–	1.1	0.63	–	4.3
	–	–	–	–	–	–	0.62	–	4.4
	–	–	–	–	–	1.1	0.63	–	4.5
	7.7	7.1	4.8	3.7	–	–	–	–	D.1
	7.6	–	4.8	–	–	–	–	–	D.2
	7.6	7.1	4.7	–	–	–	–	–	D.3
	>5.7	–	4.6	3.7	3.4	–	–	–	D.5
	7.6	7.0	4.7	–	–	–	–	–	D.6
	7.7	7.0	4.7	3.8	–	–	–	–	D.7

Table D.1-- Continued

Strain	I	II	III	IV	V	VI	VII	VII	Figure
						Chromosome number			
H-3									
	-	-	-	-	-	1.1	0.61	-	D.10
	-	-	-	-	-	-	0.61	-	D.11
H-4									
	7.9	4.9	-	-	-	-	-	-	4.1
	7.8	4.8	-	-	-	-	-	-	D.2
	7.8	4.7	-	-	-	-	-	-	D.3
	-	-	-	0.42	0.38	-	-	-	D.11
H-9									
	7.6	7.0	4.9	-	-	-	-	-	4.1
	>5.7	-	4.5	3.7	3.3	-	-	-	4.2
	-	-	-	-	-	1.1	0.63	-	4.3
	-	-	-	-	-	-	0.63	-	4.4
	-	-	-	-	-	1.1	0.63	-	4.5
	7.7	7.1	4.8	3.7	-	-	-	-	D.1
	7.6	-	4.8	-	-	-	-	-	D.2
	7.6	7.1	4.7	-	-	-	-	-	D.3
	>5.7	>5.7	4.7	3.7	-	-	-	-	D.4
	>5.7	-	4.6	3.7	3.4	-	-	-	D.5
	7.7	7.0	4.7	3.8	-	-	-	-	D.7
	-	-	-	-	-	1.1	0.62	-	D.10
	-	-	-	-	-	-	0.61	-	D.11
H-12									
	7.9	4.9	-	-	-	-	-	-	4.1

Table D.1-- Continued

Strain	I	II	III	Chromosome number					Figure
				IV	V	VI	VII	VII	
H-12									
	>5.7	4.5	3.7	-	-	-	-	-	4.2
	-	-	-	0.47	0.38	-	-	-	4.4
	-	-	-	0.46	0.39	-	-	-	4.5
	7.8	4.8	-	-	-	-	-	-	D.2
	7.8	4.7	-	-	-	-	-	-	D.3
	>5.7	4.7	3.7	-	-	-	-	-	D.4
	>5.7	4.6	3.7	-	-	-	-	-	D.5
	-	-	-	0.46	0.38	-	-	-	D.10
	-	-	-	0.46	0.38	-	-	-	D.11
H-25B									
	>5.7	-	4.5	3.7	3.3	-	-	-	4.2
	-	-	-	-	-	1.1	0.63	-	4.3
	-	-	-	-	-	-	0.63	-	4.4
	-	-	-	-	-	1.1	0.63	-	4.5
	7.7	7.0	4.8	3.7	-	-	-	-	D.1
	7.6	-	4.8	-	-	-	-	-	D.2
	>5.7	>5.7	4.7	3.7	-	-	-	-	D.4
	>5.7	-	4.6	3.7	3.4	-	-	-	D.5
	7.7	7.0	4.7	3.8	-	-	-	-	D.7
	-	-	-	-	-	1.1	0.62	-	D.10
	-	-	-	-	-	-	0.61	-	D.11

Table D.1-- Continued

				Chromosome number					
Strain	I	II	III	IV	V	VI	VII	VII	Figure
H-36									
	-	-	-	-	-	0.77	0.63	-	4.3
	-	-	-	-	-	0.75	0.63	-	4.4
	>5.7	>5.7	4.7	3.7	3.3	-	-	-	D.4
	7.6	7.0	4.7	-	-	-	-	-	D.8
	-	-	-	-	-	0.77	0.63	-	D.10
H-46									
	7.9	4.9	-	-	-	-	-	-	4.1
	>5.7	4.5	3.7	-	-	-	-	-	4.2
	-	-	-	0.52	0.47	0.43	0.27	-	4.4
	-	-	-	0.52	0.47	0.41	0.27	-	4.5
	7.8	4.8	3.7	-	-	-	-	-	D.1
	7.8	4.8	-	-	-	-	-	-	D.2
	7.8	4.7	-	-	-	-	-	-	D.3
	>5.7	4.7	3.7	-	-	-	-	-	D.4
	7.8	4.7	-	-	-	-	-	-	D.6
	7.8	4.7	-	-	-	-	-	-	D.7
	-	-	-	0.49	0.46	0.42	0.27	-	D.10
	-	-	-	-	-	-	0.27	-	D.11
H-48									
	7.9	4.9	-	-	-	-	-	-	4.1
	>5.7	4.5	3.7	-	-	-	-	-	4.2

Table D.1-- Continued

Strain	I	II	III	Chromosome number					Figure
				IV	V	VI	VII	VII	
H-48									
	-	-	-	0.46	0.43	0.40	-	-	4.4
	-	-	-	0.46	0.43	0.40	-	-	4.5
	7.8	4.8	-	-	-	-	-	-	D.2
	7.8	4.7	-	-	-	-	-	-	D.3
	>5.7	4.7	3.7	-	-	-	-	-	D.4
	>5.7	4.7	3.7	-	-	-	-	-	D.5
	7.8	4.7	-	-	-	-	-	-	D.7
	-	-	-	0.46	0.43	0.40	-	-	D.10
	-	-	-	0.45	0.43	0.40	-	-	D.11
180269									
	7.9	4.9	-	-	-	-	-	-	4.1
	-	-	-	0.43	0.41	-	-	-	4.4
	-	-	-	0.45	0.41	0.39	-	-	4.5
	7.8	4.8	-	-	-	-	-	-	D.2
	7.8	4.7	-	-	-	-	-	-	D.3
	>5.7	4.6	3.7	-	-	-	-	-	D.5
	7.8	4.7	3.7	-	-	-	-	-	D.6
	7.8	4.7	3.8	-	-	-	-	-	D.7
	-	-	-	0.44	0.42	0.39	-	-	D.10
	-	-	-	0.43	0.41	0.39	-	-	D.11

Table D.1-- Continued

| Strain | I | II | III | Chromosome number | | | | | Figure |
				IV	V	VI	VII	VII	
226802									
	7.9	4.9	-	-	-	-	-	-	4.1
	>5.7	4.5	3.7	-	-	-	-	-	4.2
	-	-	-	0.46	0.42	0.39	0.37	-	4.4
	-	-	-	0.44	0.42	0.39	-	-	4.5
	7.7	4.8	3.7	-	-	-	-	-	D.1
	7.8	4.8	-	-	-	-	-	-	D.2
	7.8	4.7	-	-	-	-	-	-	D.3
	>5.7	4.7	3.7	-	-	-	-	-	D.4
	>5.7	4.6	3.7	-	-	-	-	-	D.5
	7.8	4.7	-	-	-	-	-	-	D.7
Maram									
	>5.7	-	4.5	3.7	2.8	-	-	-	4.2
	-	-	-	-	-	-	0.63	-	4.3
	-	-	-	-	-	-	0.63	-	4.4
	>5.7	>5.7	4.7	3.7	2.8	-	-	-	D.4
	7.6	7.0	4.7	-	-	-	-	-	D.8
	-	-	-	-	-	-	0.62	-	D.10
OCO									
	>5.7	-	4.7	3.7	2.8	-	-	-	4.2
	-	-	-	-	-	-	0.65	-	4.3
	7.6	7.0	4.7	-	-	-	-	-	D.6
	7.6	7.0	4.7	-	-	-	-	-	D.8

Table D.1-- Continued

Strain	I	II	III	Chromosome number					Figure
				IV	V	VI	VII	VII	
IMB-3									
	>5.7	-	4.5	3.7	3.3	-	-	-	4.2
	-	-	-	-	-	1.1	0.63	-	4.3
	7.6	7.0	4.7	-	-	-	-	-	D.6
	7.6	7.0	4.7	-	-	-	-	-	D.8
LP-1									
	7.6	7.0	4.9	-	-	1.6	-	-	4.1
	>5.7	-	4.5	3.7	3.3	1.6	-	-	4.2
	-	-	-	-	-	-	0.63	-	4.3
	-	-	-	-	-	-	0.63	-	4.4
	-	-	-	-	-	-	0.63	-	4.5
	7.7	7.1	4.8	3.7	-	1.6	-	-	D.1
	>5.7	>5.7	4.7	-	-	1.6	-	-	D.4
	>5.7	-	4.6	3.7	3.4	1.6	-	-	D.5
	7.6	7.0	4.7	3.7	-	-	-	-	D.6
	7.6	7.0	4.7	-	-	1.6	-	-	D.7
	-	-	-	-	-	-	0.61	-	D.10
	-	-	-	-	-	-	0.60	-	D.11

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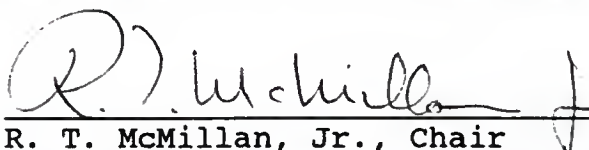Woodcock, D. M., Crowther, P. J., Doherty, J., Jefferson, S., DeCruz, E., Noyer-Weidner, M., Smith, S. S.,

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BIOGRAPHICAL SKETCH

The author was born on November 5, 1958, in Galle, the capital of southern Sri Lanka. He entered the University of Peradeniya, Sri Lanka in 1978 and received a B.S. degree in agriculture with honors in 1982. After four years of work as an assistant lecturer in the University of Ruhuna, Sri Lanka, he entered the University of Florida, Gainesville, to pursue graduate studies. He received an M.S. degree in plant pathology in 1989 and continued in the Ph.D program in molecular plant pathology.
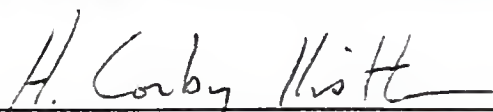
His goal is to continue research in molecular plant pathology.

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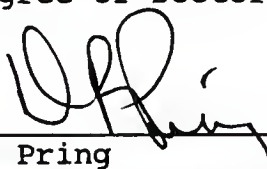
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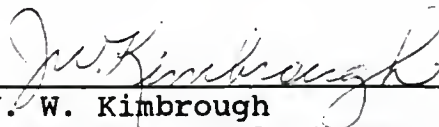
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This dissertation was submitted to the Graduate Faculty
of the College of Agriculture and to the Graduate School and
was accepted as partial fulfillment of the requirements for
the degree of Doctor of Philosophy.

August, 1992

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